CHARACTERISTICS OF PROTEASES FROM STOMACHLESS AQUATIC ORGANISMS

By

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Proteases were partially purified from the pyloric ceca of mullet (*Mugil cephalus*) and hepatopancreas from Louisiana swamp crawfish (*Procambarus clarkii*) by chromatographic procedures.

Partially purified mullet proteases were effective in inactivating commercial orange pectinesterase at room temperature.

Four trypsin-like enzymes, purified from the hepatopancreas of crawfish, were designated as CP-I, CP-II, CP-III and CP-IV in order of elution following DEAE-Sepharose chromatography. These were inhibited by protease inhibitors such as phenyl methyl sulfonyl fluoride, soybean trypsin inhibitor, aprotinin, tosyl lysine chloromethyl ketone but not

by the chymotrypsin inhibitor tosyl phenylalanine chloromethyl ketone. Therefore, these crawfish proteases could be considered as serine-type proteases and classified as trypsin-like. The apparent molecular weights of CP-I, CP-II, CP-III and CP-IV were determined to be 35.0, 41.2, 37.9 and 39.5 kDa, respectively, using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The proteases had optimal esterase activity at pH 8.0-8.5 and at a temperature between at 60-70°C. Crawfish proteases were rich in acidic amino acids. Activation energies for hydrolysis of tosyl arginine methyl ester by crawfish proteases were 6.98 - 8.34 kcal/mole. Unlike other serine proteases, the activities of CP-I and CP-II were activated by mercury chloride ($HgCl_2$) while CP-III and IV were inhibited.

Immunological study showed that the four crawfish proteases are crossreactive and, therefore, they have shared structural components. Crawfish proteases were able to inactivate orange peel pectinesterase, tomato pectinesterase and pectate lyase c at room temperature.

Using pectate lyase c from *Erwinia chrysanthemi* as a model protein substrate of known sequence and three-dimensional structure, the peptide fragmentation patterns generated by each crawfish proteases was determined by Matrix Assisted Laser Desorption Time-of-flight mass spectrometry.

Cleavage sites in native substrate were at lysine residue. The pectate lyase c cleavage hydrolyzed by crawfish proteases were similar to each other but clearly different from trypsin.

INTRODUCTION

An enzyme has been defined as a protein with catalytic properties due to its power of specific activation (Dixon and Webb, 1979). Enzymes have been used as processing aids in the manufacture of food products to improve their quality, solubility and stability for centuries (Whitaker, 1994a; Maugh, 1984). About 50% of the enzymes used as industrial processing aids are protein hydrolases which have been used in a number of industrial applications including laundry detergents, feed, leather treatment, silk degumming, cheese making, chill proofing, meat tenderizing, fermented sauces, and the production of pharmaceuticals (Maugh, 1984).

While almost any living organism can be considered a potential source of useful enzymes, only a limited number of plant and animal tissues are economic sources and the greatest diversity of these comes from microorganisms (Godfrey and Reichelt, 1983).

Enzymes derived from plant sources and used extensively in the food industry include papain, bromelin, ficin, and amylases. Animal enzymes of economic importance include trypsins, lipases, and gastric proteases. Proteolytic enzymes

obtained from livestock offal currently cannot meet the demand, resulting in increasing interest in microbial enzymes. Furthermore, the future availability of traditional enzyme sources will be dependent on the political and agricultural policies that control the production of livestock for slaughter (Godfrey and Reichelt, 1983). Moreover, traditional animal enzyme sources have been restricted to relatively few species, namely, bovine and porcine offal.

Recently, interest has developed in the proteases found in stomachless marine organisms. It was reported that trypsins and chymotrypsins from higher vertebrates do not hydrolyze efficiently native proteins while proteases from stomachless species hydrolyze native proteins (Jany, 1976; Mihalyi, 1978). The acid environment of the stomach assists denaturation and hydrolysis and enhances the efficiency of protein hydrolysis by these intestinal enzymes. However, in stomachless aquatic organisms, like mullet and crawfish, the digestion of proteins in the absence of acid denaturation and gastric proteases is compensated for by intestinal enzymes (trypsin, chymotrypsin and other digestive proteases or peptidases) that are more efficient in digesting native proteins (Simpson and Haard, 1987b). Pfleiderer et al. (1967) provided additional evidence by demonstrating that a protease from crayfish was able to

hydrolyze native ribonuclease and inactivate native lactate dehydrogenase while bovine trypsin had no effect.

In this study it was hypothesized that digestive enzymes from stomachless aquatic organisms might have sufficiently unique properties for their use over conventional sources of proteases in food processing operations. For example, if the proteases from stomachless aquatic organisms could inactivate native deleterious enzymes, they could be used to produce minimally processed juice products like "Fresh Squeezed Orange Juice" (FSOJ).

The loss of cloud in citrus juices during storage and distribution is one of the quality problems in limiting a FSOJ product. Cloud, the suspended material present in citrus juices, is an unstable colloidal system. Breakdown of cloud adversely affects the appearance and consumer's acceptability of the product. It has been determined that the enzyme pectinesterase (PE) is the cause of cloud loss. Traditionally, PE is inactivated by thermal processing, lowering the pH, addition of HCl and by ion exchange treatment. However, these treatments bring about undesirable changes in color, loss of flavor and odor, and a decrease in vitamin C content of the juice. Alternative methods are needed to avoid these undesirable changes.

The objectives of this study were as follows:

1) To purify proteases from pyloric ceca of mullet (*Mugil cephalus*) and to evaluate their effect on orange pectinesterase.
2) To establish purification methods and characterize the proteases from hepatopancreas of crawfish (*Procambarus clarkii*).
3) To evaluate the effect of crawfish proteases on other native enzymes such as orange pectinesterase, tomato pectinesterase and pectate lyase c.
4) To determine the cleavage patterns of crawfish proteases compared to bovine trypsin using native and denatured pectate lyase c as a target protein.

LITERATURE REVIEW

Enzyme Applications

The area of enzymology has continued to grow rapidly for more than 60 years because of its importance to a large segment of the sciences, especially biochemistry, physical chemistry, microbiology, genetics, botany, zoology, food science, nutrition, pharmacology, toxicology, pathology, physiology, medicine, and chemical engineering. Enzymes have been used in practical applications as diverse as brewing and industrial fermentations, pest control and chemical warfare, dry cleaning, sizing and detergents, analytical determinations, and recombinant DNA technology (Whitaker, 1994a). A revolution in biotechnology with the growing understanding of gene technology has had a profound impact on the enzyme industry. Techniques such as genetic engineering and protein engineering are used for mass production and to adjust the properties of the enzyme (Novo Nordisk, 1995).

Industrial Uses

Enzymes are used in industry according to their functions: (1) reduce viscosity, (2) improve extractions, (3) make bioconversions, (4) cause separations, (5) change functionality, (6) synthesize chemicals, and (7) improve cleaning (Boyce, 1986).

Massive quantities of enzymes are used in detergents. To most people, the best known application of enzymes is in the manufacture of enzymatic washing agents (Novo Nordisk, 1995). Amylases are used to remove stains of starchy foods such as mashed potatoes and spaghetti. Proteases remove protein stains such as grass, blood, egg and human sweat. Oily fatty stains can be removed by lipases. Cellulases are able to modify the structure of cellulose fibrils such as those found on cotton and cotton blends, affecting color brightening, softening, and particulate soil removal.

Considerable efforts have been devoted to applying enzymes in the wood processing industry. One important application is the removal of pitch which tends to coat machinery and cause interruptions in production, and decrease paper quality by causing discoloration, from wood prior to or during pulping. Novo Nordisk Nordisk produces a liquid lipase preparation for addition to the pulp slurry to reduce pitch deposit. Lipases are also beginning to be used to remove the

ink from used paper prior to recycling (Cheetham, 1995; Novo Nordisk, 1995).

Enzymes are being used increasingly in textile processing. When fabrics are made from cotton or cotton blends, the longitudinal threads have to be coated with an adhesive "size" (usually starch or related substance) to prevent them from breaking during weaving. The size must then be removed before processes such as dyeing and crease-proofing (Novo Nordisk, 1994). Bacterial amylases are most useful because desizing can be accelerated by operating at elevated temperatures and because of the resistance of the bacterial amylase to inhibitors and high temperature (Cheetham, 1995).

In the leather industry, enzymes are used in bating, soaking, unhairing and degreasing. Hides and skins contain proteins and fat between the collagen fibers, which must be partially or totally removed before tanning. The proteins can be removed by proteases and the fat removed by lipases (Novo Nordisk, 1995).

Enzymes are used in medical and pharmaceutical areas. Asparaginase is used for treating leukemia and disseminating cancers which require asparagine for growth. Trypsin or collagenase is used to remove dead tissue from wounds, burns, ulcers, etc. so as to speed the growth of new tissue and skin grafts, as well as to inhibit the growth of some contaminant

organisms (Cheetham, 1995). Enzymes have given promising results in the treatment of blood clots (thrombosis). The most widely used enzymes for this purpose are urokinase prepared from human urine, and streptokinase produced by certain bacteria (Novo Nordisk, 1995). Enzymes are also being utilized for clinical analyses.

Enzymes in Food Processing

Enzymes play an important role in the food industry in both traditional and novel products. Enzymes are very important to the food industry because they are capable of specifically manipulating all the major biological macromolecules, proteins, carbohydrates, lipids and nucleic acids, as well as smaller molecules such as amino acids, sugars and vitamins. Some of these enzymes will be active during and after processing, which could be either advantageous or deleterious to the processing (Taylor and Leach, 1995).

Table 1 gives a summary of some of the beneficial as well as detrimental activities of enzymes in foods. What may be classified as an undesirable enzymatic activity in one raw material may be beneficial in another. An appropriate example is polyphenol oxidase which causes marked losses in bananas, apples, and potatoes because of the browning reaction while it

Table 1. Some uses and suggested uses of enzymes in foods and food processing

Enzyme	Food	Purpose or action
Amylase	Baked goods	Increase in maltose content for yeast fermentation.
	Brewing	Conversion of starch to maltose for fermentation. Removal of starch turbidities.
	Cereals	Conversion of starch to dextrins and maltose. Increase in water absorption.
	Chocolate/cocoa	Liquefication of starches for free flow.
	Confectionery	Recovery of sugars from candy scraps.
	Fruit juices	Removal of starches to increase sparkling properties.
	Jellies	Removal of starches to increase sparkling properties.
	Pectin	Aid in preparation of pectin from apple pomace.
	Syrups and sugars	Conversion of starches to low-molecular-weight dextrins (corn syrup).
	Vegetables	Hydrolysis of starch as in tenderization of peas.
Cellulase	Brewing	Hydrolysis of complex carbohydrate cell walls.
	Coffee	Hydrolysis of cellulose during drying of beans.
	Fruits	Removal of graininess of pears, peeling of apricots, tomatoes.
Dextran-sucrase	Sugar syrups	Thickening of syrup.

Table 1 (Continued)

Enzyme	Food	Purpose or action
Dextran-sucrase	Ice cream	Addition of dextran as thickening agent, for body.
Invertase	Artificial honey	Conversion of sucrose to glucose and fructose.
	Candy	Manufacture of chocolate-coated soft-cream candies.
Lactase	Ice cream	Prevention of crystallization of lactose, which results in grainy, sandy texture.
	Feeds	Conversion of lactose to galactose and glucose.
	Milk	Stabilization of milk proteins in frozen milk by removal of lactose. Hydrolysis of lactose, permitting use by adults deficient in intestinal lactase and infants with congenital lactase deficiency.
Tannase	Brewing	Removal of polyphenolic compounds.
Pentosanase	Milling	Recovery of starch from wheat flour.
Naringinase	Citrus	Debittering citrus juice by hydrolysis of the glucoside, naringin.
Pectic enzymes Useful	Chocolate/ cocoa	Hydrolytic activity during fermentation of cocoa.
	Coffee	Hydrolysis of gelatinous coating during fermentation of beans.
	Fruits	Softening.
	Fruit juices	Improving yield of press juices, preventing cloudiness, improving concentration processes.
	Olives	Extracting oil.
	Wines	Clarification.

Table 1 (Continued)

Enzyme	Food	Purpose or action
Pectic enzymes Deteriorative	Citrus juice	Destruction and separation of pectic substances.
	Fruits	Excessive softening action.
Proteases Useful	Baked goods	Softening action in doughs. Cut mixing time, increase extensibility of doughs. Improvement in grain, texture, loaf volume. Liberate α-amylase.
	Brewing	Body, flavor, and nutrient development during fermentation. Aid in filtration and clarification, chill-proofing.
	Cereals	Modification of proteins to increase drying rate, improve product handling characteristics. Manufacture of mise and tofu.
	Cheese	Casein coagulation. Characteristic flavors during aging.
	Chocolate/ cocoa	Action on beans during fermentation. Improve drying properties.
	Egg/ egg products	Improve drying properties.
	Feeds	Use in treatment of waste products for conversion to feeds.
	Meats and fish	Tenderization. Recovery of protein from bones, trash fish. Liberation of oils.
	Milk	In preparation of soybean milk.
	Protein hydrolysates	Condiments such as soy sauce and tamari sauce. Specific diets. Bouillon, dehydrated soups, gravy powders, processed meats.

Table 1 (Continued)

Enzyme	Food	Purpose or action
Proteases Useful	Wines	Clarification.
Proteases Deteriorative	Eggs	Shelf life of fresh and dried whole eggs.
	Crab, lobster	Overtenderization if not inactivated rapidly.
Proteases Deteriorative	Flour	Influence on loaf volume and texture if too active.
Lipase Useful	Cheeses	Aging, ripening, and general flavor characteristics.
	Oils	Conversion of lipids to glycerol, fatty acids, and monoglycerides
	Milk	Production of milk with slightly cured flavor for use in milk chocolates.
Lipase Deteriorative	Cereals	Overbrowning of oat cakes. Brown discoloration of wheat bran.
	Milk and dairy products	Hydrolytic rancidity.
	Oils	Hydrolytic rancidity.
Phosphatases	Baby foods	Increase available phosphate.
	Brewing	Hydrolysis of phosphate compounds.
	Milk	Monitoring progress of pasteurization.
Nucleases	Flavor enhancers	Production of nucleotides and nucleosides.
Peroxidases Useful	Vegetables, fruits	Monitoring progress of blanching.
	Glucose detection	In combination with glucose oxidase.

Table 1 (Continued)

Enzyme	Food	Purpose or action
	Doughs	Cross-linking of proteins
Peroxidases Deteriorative	Fruits	Contribution to browning action.
Catalase	Milk	Destruction of H_2O, in cold pasteurization.
	Variety of products	To remove glucose and/or oxygen to prevent browning and/or oxidation. Used in conjunction with glucose oxidase
	Vegetables	Monitoring progress of blanching.
Glucose oxidase	Variety of products	Removal of oxygen and/or glucose from products such as beer, cheese, carbonated beverages, dried eggs, fruit juices, meat and fish, milk powder, wine to prevent oxidation and/or browning. Used in conjunction with catalase.
	Glucose determination	Specific determination of glucose. Used in conjunction with peroxidase.
Polyphenol oxidase Useful	Tea, coffee, prunes, unbleached raisins	Development of browning during ripening, fermentation, and/or aging process.
Polyphenol oxidase Deteriorative	Fruits, vegetables	Browning, off-flavor development, loss of vitamins.
Lipoxygenase	Vegetables	Destruction of essential fatty acids and vitamin A, development of off-flavors.
Ascorbic acid oxidase	Vegetables, fruits	Destruction of vitamin C (ascorbic acid).
Thiaminase	Meats, fish	Destruction of thiamine.

Adapted from Whitaker (1994a).

is necessary for the development of proper color in tea, coffee, unbleached raisins and prunes (Whitaker, 1994a).

When enzymes are considered for use in a food process, it is essential to ensure that they will confer some commercial benefit. Enzymes may improve the conversion of raw material such as in the hydrolysis of starch to glucose. Acid hydrolysis of starch gives limited conversion whereas enzymes can improve the conversion yield (Woods and Swinton, 1995). In the traditional brewing process, malt acts both as a raw material providing starch and protein, and as a source of enzymes. Considerable savings can be made by replacing at least part of the malt by industrial enzymes and unmalted cereals such as barley (Novo Nordisk, 1995; Taylor and Leach, 1995).

Much of the recent effort in improving enzyme uses in food processing has been focused on the production of large quantities of enzymes at economically affordable costs. Recombinant chymosin is a well-known example. The eukaryotic gene of chymosin is cloned into and expressed by microorganisms and the enzyme can be produced by fermentation. The recombinant enzyme is commercially obtained from *Escherichia coli*, *Kluyveromyces lactis*, and *Aspergillus awamori* (Dornenburg and Lang-Hinrichs, 1994).

Many ongoing investigations are directed to modify individual enzymes for specific functional properties. Enzymes used in food processing can be tailored to increase the efficiency of the process and ultimately lower the cost of operations. For example, the enzymes, α-amylase and glucoamylase in corn syrup production can be improved to benefit the industry and ultimately the consumer (Wong, 1995).

Protease Uses

More than 80% of all industrial enzymes are hydrolytic in action. Almost 60% of hydrolase enzymes are proteolytic. Proteases degrade protein molecules by hydrolyzing peptide bonds. Proteases are used most extensively where they act to improve the quality, stability or solubility of foods, as in baking, brewing, cheesemaking and also meat processing (Godfrey and Reichelt, 1983).

The use of rennet (both animal and microbial) in the manufacture of cheese stands out as, economically, the most important use of proteases in the food industry (Adler-Nissen, 1996). This enzyme, by producing a single cleavage between phenylalanine and leucine in the casein of milk, results in clotting, which is the essential process for cheese-making (Law and Goodenough, 1995).

Proteases are the most widely used enzymes in the detergent industry. They remove protein stains such as grass, blood, egg and human sweat. These organic stains have a tendency to adhere strongly to textile fibers. The proteins act as glues, preventing the water-borne detergent systems from removing some of the other components of soiling, such as pigments and street dirt. Proteases hydrolyze proteins and break them down into more soluble polypeptides or free amino acids (Novo Nordisk, 1995).

The rheological characteristics of wheat flour dough are dependent upon the gluten protein they contain. This protein forms a network during dough preparation responsible for the mechanical loading ability it exhibits (Reichelt, 1983). In the baking industry, proteases are used for softening in dough, reducing mixing time, increasing extensibility, and improving texture and grain loaf volume (Simpson and Haard, 1987a).

Papain has been used in beer industry for the prevention of haze formation during cold storage of the beer, the so-called "chillproofing" of beer. The effect of the enzyme is believed to be the proteolytic degradation of ill-defined complexes between proteins and tannins (Moll, 1987). Also papain is the most important enzyme used for meat tenderizing although a considerable number of enzymes have been promoted

in this area. The simplest way to apply the enzyme is to sprinkle it over slices of raw meat, but papain also may be injected into the carcass and circulated through veins (Schwimmer, 1981).

When proteases are applied to fish muscle, the desired result is generally a through degradation of the fish muscle into soluble peptides. The traditional autolytic processes leading to the various fish sauces of Southeast Asia exemplify this application (Beddows and Ardeshir, 1979).

Proteases are widely used to modify proteins for making better products. For flavor enhancement, proteases are used to hydrolyze proteins because the more amino acids and small peptides created, the more flavor (Novo Nordisk, 1995). Protein hydrolysates produced by controlled enzymatic hydrolysis in the food industry are considered a new class of food ingredients with interesting functional properties (Adler-Nissen, 1996).

Proteases from Aquatic Organisms

Proteases from Aquatic Organisms

Digestive proteases have been purified and characterized from various aquatic organisms such as anchovy, capelin, cod, sardine, krill, shrimp, starfish and trout.

Martinez et al. (1988) purified two trypsin-like enzymes from the pyloric caeca and intestine of anchovy (*Engraulis encrasicholus*) by ammonium sulfate fractionation (30-70% saturation) and chromatographic separation. The molecular weights (MW) of the enzymes were 27 kDa and 28 kDa, respectively. Their isoelectric points were about 4.9 and 4.6 and they had similar amino acid composition. Optimum pH of the enzymes was 8-9 for the hydrolysis of N-benzoyl-DL-arginine-p-nitroanilide (BAPA). Tosyl lysine chloromethyl ketone (TLCK) inhibited completely the activity while phenyl-methyl-sulphonyl-fluoride (PMSF), benzamidine and soybean trypsin inhibitor (SBTI) were also effective inhibitors.

Two trypsin-like enzymes were purified from the gut of the Arctic fish capelin (*Mallotus villosus*) (Hjelmeland and Raa, 1982). Both enzymes had a molecular weight of about 28 kDa as determined by SDS-electrophoresis. Their isoelectric points were 5.6-5.9 and 5.1-5.3, respectively. The enzymes had a pH optimum of 8-9 with BAPA and their stability was not affected by $CaCl_2$. Even though capelin is an Arctic fish having environmental temperatures below 4°C, the temperature optimum (42°C) for the enzymes was not much different from those from warm-blooded animals. Both enzymes were completely inhibited by aprotinin, soybean trypsin inhibitor (SBTI), and tosyl lysine chloromethyl ketone (TLCK), and partially by

phenyl methyl sulfonyl fluoride (PMSF), whereas chymotrypsin inhibitor tosyl phenylalanine chloromethyl ketone (TPCK) had no effect. The stability of the enzymes was not affected by $CaCl_2$.

Serine proteases from Atlantic cod (*Gadus morhua*) have been studied (Shin and Zall, 1986; Raae and Walther, 1989; Ásgeirsson et al., 1989; Simpson et al., 1989a and 1990). Shin and Zall (1986) purified and characterized a protease from the pyloric caeca of cod. It had a molecular weight of 24 kDa and optimal pH of 9.0-9.6 with casein and hemoglobin as substrate. This enzyme was not sensitive to calcium ion but was very sensitive to copper and silver ions--a common property of the serine protease trypsin. The enzyme was strongly inactivated in the presence of PMSF and TLCK, which suggests that it is a serine protease. Raae and Walther (1989) purified chymotrypsin, trypsin and elastase from the pyloric caeca of cod. Ásgeirsson et al. (1989) purified three trypsins (Enzyme I, II, III), and all three trypsins had a similar molecular weight of 24.2 kDa and pI's of 6.6, 6.2 and 5.5, respectively. The apparent Km (Km,app) values determined at 25°C for the predominant form (I) of Atlantic cod trypsin on p-tosyl-L-arginine methyl ester (TAME) and N-α-benzoylarginine-p-nitroanilide (BAPA) were 29 μM and 77 μM respectively, which were notably lower than those determined for bovine trypsin

(46 μM and 650 μM respectively). Simpson et al. (1989a and 1990) isolated a trypsin (MW 24 kDa) from the pyloric ceca of Atlantic cod. The enzyme hydrolyzed BAPA and TAME with apparent Km values of 1.48 mM and 0.22 mM, respectively, which are higher than those determined by Ásgeirsson et al.(1989). The pH and temperature optima with BAPA were 7.5 and 40°C, respectively. Amino acid analysis revealed that the enzyme is rich in serine, glycine, glutamate and aspartate, but poor in basic amino acid residues compared to trypsins from warm-blooded animals.

Trypsin was isolated from the pyloric ceca of Greenland cod (*Gadus ogac*) by Simpson and Haard (1984a,b). Greenland cod inhabits shallow coastal waters where temperatures remain relatively low and fairly constant (between -2° and 2°C). The enzyme (MW 23.5 kDa) showed an apparent Km of 0.14 mM at 5°C and 0.26 mM at 35°C with TAME. The apparent Km for cod trypsin with BAPA increased in specificity from 1.67 mM at 25°C to 0.90 mM at 35°C. However, the Km,app for bovine trypsin was not affected substantially by temperature. These results show that Greenland cod trypsin is a better catalyst at low temperatures. Hofer et al.(1975) determined the Km,app for trypsin from different fish that have a temperature preference of 10°-25°C and concluded that Km measured with BAPA at the temperature preference of the fish is lower for cold-water

fish than it is for fish from warm water or endothermic organisms.

Murakami and Noda (1981) purified three alkaline proteases from the pyloric ceca of sardine (*Sardinops melanosticta*). The molecular weights were 22.9 kDa, 28.7 kDa and 27 kDa, and the pIs were 5.45, 5.3 and 4.85, respectively. Two enzymes were found to be anionic and chymotrypsin-like while the third, also anionic was trypsin-like. Although these fish enzymes have properties common to those of bovine pancreatic cationic trypsin and chymotrypsin, they were distinctly different in their optimum pH, pH stability, net charge and immunological properties.

Osnes and Mohr (1985) reported three enzymes from Antarctic krill (*Euphausia superba*) capable of hydrolyzing TAME. The enzymes had molecular weights of 30 kDa (Enzyme I), and 31 kDa (Enzymes II and III), respectively. Optimum pH was about 8.2 for all three enzymes. The enzymes were inhibited by PMSF, TLCK and SBTI, whereas TPCK had no effect on activity. Therefore, they were classified as trypsin-like enzymes.

Gates and Travis (1969) purified trypsin from the hepatopancreas of white shrimp (*Penaeus setiferus*). It had a molecular weight of 24 kDa and was inhibited by diisopropyl fluorophosphate (DFP) and SBTI, but not TPCK. Shrimp trypsin had an acidic isoelectric point and no requirement for Ca^{2+} on

stability and activity of the enzyme. The optimum pH was found to be rather broad, occurring over the range of 7.0 - 9.0. Maximum proteolytic activity occurred at 49°C. Galgani et al. (1984 and 1985) purified a trypsin from *Penaeus japonicus*. The molecular weight was 25 kDa by SDS-PAGE. Optimum pH was found to be around 8.0 - 8.3 and the enzyme was stable for several days at pH 8.0. The tryptic hydrolysis of TAME was affected by standard trypsin inhibitors such as PMSF, TLCK and SBTI but not TPCK. Jiang et al. (1991) reported three trypsin-like enzymes (A,C and D) and one chymotrypsin-like enzyme (B) purified from the digestive tract (stomach and intestine) of grass shrimp, *Penaeus monodon*. Optimal temperature for hydrolysis of casein by proteases A, B and C was 65°C while D was 55°C. Molecular weights of the proteases were 18.5 kDa (A), 20.9 kDa (B), 23.3 kDa (C) and 50.1 kDa (D). Optimal pH for hydrolysis of TAME by proteases A and C was 8.0 while D was 7.0. The optimal pH of protease B for hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) was 8.0. Enzymes A and C were completely inhibited by TLCK while enzyme D was inhibited partially. Enzyme B was inhibited partially by TPCK.

Two trypsin-like enzymes were purified from starfish, *Dermasterias imbricatamby* (Camacho et al., 1970). The enzymes hydrolyzed BAPA at rates that were 2- and 1.2-fold faster than bovine pancreatic trypsin. Molecular weights of the enzymes by

gel filtration were 25 kDa to 26 kDa. Hydrolysis of BAPA by each enzyme was optimal at pH 8.0 to 8.5. Both starfish enzymes were inhibited by di-isopropyl fluorophosphate (DFP) and TLCK. However, SBTI had no effect on either of the starfish proteases. Winter and Neurath (1970) purified a trypsin-like enzyme from the starfish, *Evasterias trochelii*. It was anionic at neutral pH, unstable below pH 5, and did not require calcium ions for stability. The enzyme was inhibited by DFP and TLCK; however it was also completely inhibited by SBTI which had no effect on the enzyme from *Dermasterias imbricata*.

Kristjánsson (1991) isolated a trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). The molecular weight of the enzyme was estimated as 25.7 kDa by SDS-PAGE. The enzyme was inhibited by PMSF and TLCK but not TPCK. Trypsin inhibitors, benzamidine, SBTI, aprotinin, and Bowman-Birk inhibitor, all inhibited the enzyme, confirming its identity as a trypsin. Apparent Km against BAPA was 0.077 mM at 20°C which was lower than that for bovine trypsin (0.46 mM).

Proteases from Stomachless Organisms

Stomachless fish lack a distinct stomach and do not possess the pepsin-HCl system. According to Beauvalet (1933),

intestinal secretions from stomached fish are not adequate in themselves to digest protein, whereas intestinal secretions from stomachless fish are sufficient to facilitate complete protein digestion. This observation is consistent with the view that such fish secrete enzymes that hydrolyze native proteins efficiently. Digestion of protein in the intestines is generally assumed to be preceded by acid denaturation and acid proteolysis in the stomach. Previous studies by Jany (1976) and Pfleiderer et al. (1967) have indicated that intestinal enzymes from higher vertebrates do not hydrolyze native proteins appreciably. However, proteases from stomachless fish such as crayfish and cunner are able to hydrolyze native proteins (Simpson and Haard, 1987b; Simpson et al.1989b; Pfleiderer et al., 1967). The unique properties of these enzymes could be used in various food processing applications.

Simpson et al. (1989b) recovered trypsins from Atlantic cod, Greenland cod, and the stomachless cunner and compared their properties with those of commercially available bovine trypsin. The relative amidase activities with BAPA as substrate of the trypsins were 130 to 200% higher for the fish enzymes than for their bovine counterpart. The activity of cunner trypsin was about 2 times more active than bovine trypsin. The relative esterase activities toward TAME were 110

to 120% higher for the fish trypsins than that of bovine trypsin. The cunner trypsin showed about a 1.1-fold increase in esterase activity compared with bovine trypsin. Cunner trypsin hydrolyzed the native protein substrates, hemoglobin and ribonuclease A, 1.7 times faster than bovine trypsin and 2 times faster than Atlantic cod trypsin. For the substrate specificity (catalytic efficiency), defined as the ratio Vmax/K'm, cunner trypsin was the most efficient catalyst for the amidase reaction, followed by Atlantic cod trypsin, Greenland cod trypsin, and bovine trypsin in that order.

Pfleiderer et al.(1967) reported a trypsin-like enzyme (MW 24 kDa) from crayfish. In contrast to mammalian trypsin, the crayfish (*Astacus fluviatilis* Fabr.) trypsin-like enzyme hydrolyzed native ribonuclease A and quickly inactivated native lactate dehydrogenase.

Guizani (1991) purified a trypsin-like enzyme from the pancreas of Louisiana swamp crayfish (*Procambarus clarkii*). The crayfish enzyme had a molecular weight of 33.7 kDa, and hydrolyzed TAME and p-toluene-sulfonyl-L-lysine methyl ester (TLME) but not BAPA. The activity of the enzyme with TAME was more than twice that with TLME as substrate. This crayfish protease inactivated commercial pectinesterase.

Trypsin (MW 24 kDa) was purified and characterized from mullet (*Mugil cephalus*) by Guizani et al. (1991). The enzyme

hydrolyzed BAPA and TAME and it was inhibited by PMSF, SBTI, aprotinin and benzamidine. Optimum pH and temperature were 8.0 and 55°C, respectively. The enzyme showed greater affinity for TAME (Km = 0.19 mM) as a substrate than for BAPA (Km = 0.49 mM), and preferential hydrolysis of ester bonds than amide bonds. Mullet trypsin was about 2 times more active than bovine trypsin towards BAPA and 1.2 times more active than bovine trypsin towards TAME at 25°C.

Application of Proteases from Aquatic Organisms

Approximately 50% of the enzymes used for industrial processes are protein hydrolases (Godfrey and Reichelt, 1983). Proteolytic enzymes are employed for such diverse uses as coagulants in cheese making, chill proofing of beer, tenderization of meat, recovery of protein from food-processing wastes, detergent aids, hide and leather treatment, and as aids in baking cereal products. Commercial supplies of food processing enzymes are presently available from various plant, animal and microbial sources.

Studies on potential use of proteases from aquatic organisms in food processing include squid fermentation (Lee et al., 1982), cheese making (Brewer et al., 1984; Shamsuzzaman and Haard, 1983, 1985), production of fish sauce from male capelin (Raksakulthai et al., 1986), prevention of

copper-induced oxidation of milk (Simpson and Haard, 1984c; Guizani, 1991), improvement of flavor extractibility from crab waste (Kim et al.,1994) and inactivation of pectinesterase in orange juice (Guizani, 1991).

Greenland cod trypsin (GCP) was applied to prevent copper-induced oxidized flavor in milk (Simpson and Haard, 1984c). Both bovine trypsin and GCP showed similar effectiveness in preventing oxidation of milk lipids. After pasteurization, GCT added to the milk was completely inactivated, whereas approximately 47% of the added bovine trypsin remained active. In the study by Guizani (1991), mullet trypsin did not survive the pasteurization, but bovine trypsin did survive the treatment and retained about 48% of its original activity. Since continued trypsin activity in milk is undesirable, fish trypsins might offer an advantage over bovine trypsin.

Pepsin was isolated from the stomach lining of Atlantic cod and its rate of milk clotting was compared with calf rennet and porcine pepsin by Brewer et al. (1984). Pepsin exhibited a relatively low ratio on milk clotting to peptic activity. However, Atlantic cod pepsin clotted milk more efficiently at low temperature than calf chymosin indicating its potential usefulness in cold renneting milk. Shamsuzzaman and Haard (1983) reported that a crude preparation of gastric

proteases from harp seal (*Pagophilus groenlandicus*) coagulated milk over a wider pH range than porcine pepsin and had a higher ratio of milk clotting to proteolytic activity with hemoglobin at pH 1.8. Cheddar cheese prepared with seal gastric protease gave significantly higher sensory scores than cheeses made with calf rennet. Chemical analysis of the cheeses revealed a lower concentration of citrate-HCl soluble nitrogen and fewer free and peptide-bound amino acids in seal gastric protease cheese than in the cheeses made with calf rennet or *Mucor miehei* protease.

Kim et al. (1994) reported that crayfish hepatopancreatic extract (CHE) with high proteolytic enzyme activity improved the extraction of volatile flavor compounds and increased the extractability of nonvolatile nitrogen compounds from crab hard tissue (CHT) obtained by mechanical separation of composite processing. Concentration of volatile flavor compounds increased significantly in CHE-treated CHT compared with those from untreated substrate but dienals and aromatic hydrocarbons decreased. Pyrazines, especially 2-ethyl-6-methylpyrazine and 2,3-dimethylpyrazine, were in markedly higher concentrations from the CHE treatment.

The fermentation of brine squid was accelerated by supplementation with Greenland cod trypsin (Lee et al., 1982). Sweetness and improved taste of the product was demonstrated

when the brine was supplemented with Greenland cod trypsin. The beneficial action of protease supplementation was associated with enhanced accumulation of taste-active amino acids (glutamic, alanine, leucine, serine lysine, arginine and proline). Similar results were observed with the fermentation of herring and squid at 10°C (Simpson and Haard, 1984c). The accumulation of soluble protein in the fermentation brine occurred more rapidly when it was supplemented with cod trypsin than it did when bovine trypsin was added.

Pectinesterase

Pectinesterase (E.C.3.1.1.11) is contained largely in citrus fruits. The enzyme is firmly associated with the cell wall fractions and is located mainly in peel, rag, and juice sac tissue (Joslyn and Pilnik, 1961). The substrate of pectinesterase (PE), the pectic polysaccharides, are a major constituent of the plant middle lamella and primary cell wall. Pectic polysaccharides are composed of a rhamno-galacturonan backbone in which partially methoxylated (1->4)-α-D-galacturonan chains are interrupted at intervals by (1->2)-α-L-rhamnose units. PE has been referred to as pectase, pectin methoxylase, pectin demethoxylase, and pectin methylesterase (Aspinall, 1980). PE removes methoxyl groups from methylated pectic substances (pectin). Most PEs are thought to initiate

attack on pectin at a position adjacent to a pre-existing free carboxyl group. In pectin, only 65-75% of the carboxyl groups are esterified so there are a number of points along the chain where the enzyme may start demethoxylating the methoxyl groups. Totally methylated polygalacturonic acid is not acted upon by PE which requires a free carboxyl group for initiation of attack (Whitaker, 1994b).

Both desirable and undesirable effects before, during or after processing may be produced by PE activity in fruits and vegetables. In fresh or underpasteurized citrus juices, low methoxyl pectin produced by PE activity is precipitated by calcium ions, resulting in cloud loss of single strength juice or gelation of frozen concentrates. Both of these are serious quality defects because of negative effects such as appearance, taste, aroma, and increased sensitivity to oxidation (Joslyn and Pilnik, 1961; Rouse and Atkins, 1952).

Of the three PE forms in Naval orange, the high molecular weight PE has been shown to be the only form which affects significantly clarification of chilled citrus juices. Therefore it is the only from which plays a role in the loss of cloud in underpasteurized citrus juices. The other forms of PE are rapidly inactivated at temperatures less than 80°C, which would be the ideal pasteurization temperature required

to prevent microbial spoilage (Bisset et al., 1953), and to inactivate peroxidase (Nath and Ranganna, 1977).

To solve the problem of juice clarification, several technological approaches have been used for inactivate pectinesterase. Thermal treatment (105-115°C) is the most common technology used for cloud stabilization. However, care must be taken to avoid changes in juice flavor by the thermal process (Cruess, 1914; Irish, 1928). Degradation of PE substrate is not frequently used. It consists of degrading soluble pectin to a low degree of methylation, with enzymes such as pectin lyase or polygalacturonase that prevent pectin precipitation by calcium through reduction of the molecular weight of pectin (Baker and Bruemmer, 1969; Baker, 1977). PE could be inactivated concomitantly with the concentration process in the Thermally Accelerated Short Time Evaporator (TASTE), by pasteurization at 90°C for one minute, or by frozen storage (-20°C) (Joslyn and Pilnik, 1961). Marketing studies have demonstrated that consumers prefer and are willing to pay for juices that are as close to fresh squeezed as possible. Therefore, any process that could produce a lower temperature processed orange juice would be a tremendous economic advantage for the citrus processing industry. Recently glycoprotein inhibitor of PE was found from kiwi by Balestrieri et. al (1990) and Castaldo et. al (1991). The kind

of inhibition on kiwi pectin PE was found to be competitive using citrus pectin as a substrate.

MALDI Mass Spectrometry

Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) is an outgrowth of the direct laser desorption mass spectrometry (LD-MS) of small organic molecules that was initially developed in the 1960s and 1970s (Moore, 1997). In 1987, MALDI-MS was introduced by Karas and Hillenkamp (1988) who demonstrated that adding a small molecular weight organic acid matrix to an analyte could overcome molecular photodissociation of the sample ions induced by direct laser irradiation of the sample. The matrix in MALDI absorbs energy from the pulsed laser beam, isolates sample molecules, and provides photoexcited acid or basic sites for ionization of sample molecules in ion/molecule collisions (Hillenkamp et al. 1991). A major advantage of MALDI-MS is that the mass range extends from the low molecular weight range up to 200,000 or greater for analysis by MS and provides high sensitivity, high throughput, and simplicity of operation (Moore, 1997; Fenselau, 1997). MALDI ionizes molecules with molecular masses of 100-1,000,000 Da. Many types of mass spectrometers are used with MALDI, including time-of-flight (TOF), Fourier transform (FT), Paul trap,

magnetic sector, sector-TOF, TOF-trap, and TOF-TOF instruments. TOF analyzers are most commonly used with MALDI. The mass accuracy of MALDI-TOF instruments is generally 0.1-0.01%, and resolution can exceed 1 part in 20,000 (Fenselau, 1997).

MALDI-MS has been used most successfully for analyzing proteins and peptides. MALDI can be combined with enzymatic reactions and protein chemistry to provide structural information at three levels - molecular masses, peptide maps, and primary structure (including sequences and post translational modifications). It has also been applied to a large variety of drugs, metabolites, and other low-molecular-weight compounds (Fenselau, 1997). In combination with time-of-flight (TOF) mass spectrometry MALDI has rapidly evolved as a valuable tool for the detection and characterization of biopolymers such as peptides, proteins, oligosaccharides (Mock et al., 1991; Stahl et al., 1991) and oligonucleotides (Butler et al., 1996; Bentzley and Johnston, 1996; Pieles et al., 1993) especially in mixtures and crude samples.

Camafeita et al. (1997a) reported a procedure developed using MALDI/TOF-MS to detect gliadins directly in food samples by observing the characteristic gliadin mass pattern (ranging from 25 to 40kDa) in food. The first attempt to quantify gluten in food by MALDI/TOF-MS based on the rapid and easy

detection of gliadins via the direct visualization of the characteristic gliadin mass pattern was reported by (Camafeita et al.,(1997b). Recently, MALDI-MS has developed into an analytical technique that has many advantages for food analysis and it could become the preferred analytical technique for the analysis of many compounds in foods (Sporns and Abell, 1996).

EFFECT OF PROTEASES FROM THE PYLORIC CECA AND SMALL INTESTINES OF MULLET(*Mugil cephalus*) ON ORANGE PECTINESTERASE

Introduction

Trypsin and trypsin-like enzymes are proteases found in the digestive tract of many organisms. Purification of these enzymes in carp (Cohen et al., 1981), catfish (Yoshinaka et al., 1984) and anchovy (Martinez et al., 1988) has been reported.

Approximately 50% of the enzymes used as processing aids in the food industry are protein hydrolases. Therefore protease purification and characterization is of industrial interest. Proteases can be used in a variety of ways to improve the quality, stability and solubility of foods such as those in the baking, brewing, cheesemaking and meat processing industries.

The stomachless organisms are deprived of the acid denaturation and acid hydrolysis which takes place in the stomach and make the protein in the feed more amenable to subsequent degradation in the intestine by proteases. It is reported that proteases from stomachless organisms are more

efficient in hydrolyzing native proteins than comparable ones from organisms having a stomach (Beauvalet, 1933).

This study describes the partial purification of proteases from pyloric ceca of mullet, a stomachless fish, and their effectiveness in inactivating orange pectinesterase.

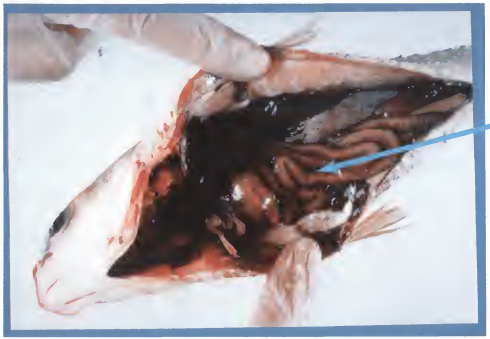
Materials and Methods

Mullet (*Mugil cephalus*) was purchased from a local seafood store and degutted (Fig.1). The pyloric ceca was removed, frozen rapidly in liquid nitrogen and ground to a fine powder in a Waring blender. The powdered pyloric ceca was stored at -70°C prior to extraction.

Extraction of Mullet Protease

Mullet powder was mixed with 1:4 (w/v), 0.05 M Tris-HCl extraction buffer containing 50 mM NaCl and 20 mM $CaCl_2$, pH 7.8, (Simpson and Haard, 1985). All steps were carried out at 4°C. The slurry was stirred overnight and centrifuged at 10,000g for 60 minutes. The supernatant was fractionated with solid ammonium sulfate and the fraction sedimenting between 30-60% saturation was collected by centrifugation at 10,000g for 60 minutes. The precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.8 and dialyzed with 10 K cut off tubing

Figure 1. Mullet showing the pyloric ceca and small intestine.



Pyloric Ceca and
Small Intestine

against 3 changes (1 L) of the same buffer overnight at 4°C. The dialyzed ammonium sulfate fraction was concentrated using an Amicon cell (Danvers, MA) with 10 K cut off filter (Pall Filtron, Northboro, MA).

Gel Filtration Chromatography

The dialyzed ammonium sulfate fraction (30-60%) after concentration was loaded onto a Sephadex G-100 column (2.5 cm id. x 30 cm). Elution was carried out with 50 mM Tris-HCl buffer, pH 7.8 at a rate of 0.2 mL/min. Fractions (4 mL/tube) were collected and then assayed for amidase activity using N-α-benzoylarginine-p-nitroanilide (BAPA, Sigma Chemical Co, Saint Louis, MO) as a substrate. Active fractions were pooled, concentrated, and loaded onto a Sephadex G-100 Superfine column (2.5 cm id. x 30 cm) under the same conditions as above. Fractions (4 mL/tube) were collected and those showing amidase activity were pooled and concentrated.

Protein Assay

The method of Braford (1976) was used for quantitative determination of protein using bovine serum albumin (Fisher Scientific Company, Fair Lawn, NJ) as a standard. Protein assay reagents were purchased from Bio-Rad Laboratories (Richmond, CA).

Protease Activity Measurements

Amidase activity was assayed based on the method of Erlanger et al. (1961) using BAPA as substrate. Two hundred microliters of the enzyme solution was added to 2.8 mL of 1 mM BAPA in 50 mM Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$. The release of p-nitroanilide was measured at 410 nm (25°C) in a DU-8 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). One unit of activity was defined as ΔA_{410nm}/min x 1000 x 3 divided by 8800, where 8800 ($M^{-1}cm^{-1}$) is the molar absorption coefficient for p-nitroanilide.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sufate-polyacrylamide gel electrophoresis(SDS-PAGE) was performed using the method of Laemmli (1970). For the enzyme purity and hydrolysis of pectinestrase by mullet protease, separations were carried out on a Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, Richmond, CA). Running gels (10 mL) were composed of 12% acrylamide, 0.3% bis, 0.1% SDS, and 0.05% ammonium persulfate in 0.375 M Tris-HCl, pH 8.8. Polymerization was initiated by addition of 0.05% N,N,N',N'-tetramethyl-ethylenediamine (TEMED). A stacking gel was composed of 4% acrylamide, 0.3% bis, 0.1% SDS, and 0.05% ammonium persulfate in 0.125 M Tris-HCl, pH 6.8. Polymerization was accomplished 1-2 hours before

sample application by addition of 0.05% TEMED. Proteins were diluted 4:1 with sample buffer (0.065 M Tris-HCl buffer, pH 6.8, containing 10% glycerol, 1% SDS, 0.005% bromophenol blue and 1% 2-mercaptomethanol) and denatured by heating at 100°C for 4 min. Proteins were then loaded onto 1 mm thick mini gels and run in a buffer of 0.6% Tris, 2.9% glycine and 0.1% SDS, pH 8.3. Current was kept constant at 120 V. Following electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 (Eastman Kodak Co., Rochester, NY), and destained in 40% ethanol and 10% acetic acid.

Pectinesterase Assay

Pectinesterase (PE) activity was measured titrimetrically. A one percent pectin solution was prepared for the standard assay in 10 mM sodium chloride and 1 mM sodium azide. This solution was adjusted to pH 7.5 at 25°C. While stirring 25 mL of the pectin solution, the PE sample was added and the initial reaction velocity was measured by automatic titration of the liberated carboxyl groups with standardized 0.01 N sodium hydroxide in a Combi recording pH-stat (Metrohm Ltd., Hrissau, Switzerland). One unit of PE activity is defined as the amount of carboxyl groups liberated per minute at standard assay conditions.

Effect of Mullet Proteases on Pectinesterase

Two different amidase activity units for mullet protease (MP 3.2 and 6.4) were mixed with 31 units of commercial orange peel PE (Sigma Chemical Co, Saint Louis, MO) and incubated at room temperature. Pectinesterase activity was measured over time (0-4 days) by the titrimetric method. After four days of incubation, PE, MP and a mixture of PE and MP were applied onto a 12% SDS gel and run as described under the SDS-PAGE section.

Thermostable PE (2.6 units) purified from orange peel using CM-Sephadex C-50, Phenyl Sepharose CL-4B and CM-Bio Gel A chromatographic procedures (Hou et al. 1997) was incubated with 1.9 and 3.8 units of MP at room temperature. Pectinesterase activity was measured over time (0-4 days) by the titrimetric method.

Results and Discussion

Purification of Mullet Proteases

The purification of mullet protease from twenty five grams of mullet pyloric ceca is summarized in Table 2. The ammonium sulfate fraction (30-60%) was loaded onto a Sephadex G-100 column and eluted with 50 mM Tris-HCl, pH 7.8. The

Table 2. Purification of mullet protease

Step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Yield (%)	Purification fold
Crude Extract[b] (10,000g)	286.4	1,187.4	4.2	100.0	1.0
30-60% $(NH_4)_2SO_4$	33.4	756.0	22.6	63.7	5.4
Sephadex G-100	14.1	629.3	44.6	53.0	10.6
Sephadex G-100 Superfine	6.8	426.4	62.7	35.9	14.9

[a] One unit catalyzes the hydrolysis of one µmol BAPA/min at 25° C, pH 8.2.
[b] Twenty five grams of mullet pyloric ceca was used for extraction.

amidase activity was found in the fraction of the second protein peak (Fig. 2). These fractions were pooled, concentrated and loaded onto a Sephadex G-100 Superfine column (Fig. 3). Fractions (21-34) having amidase activity were pooled, concentrated and considered as partially purified mullet protease. The specific activity increased from 4.2 for the crude extract to 62.7 for the Sephadex G-100 Superfine column fraction. The yield was 35.9% while the purification fold was 14.9.

Effect of Mullet Protease on Pectinesterase

Pectinesterase (PE) and thermostable pectinesterase (TSPE) were incubated with mullet protease (MP) at room temperature and the PE activity was measured with time (Fig. 4). Two different concentrations of MP (3.2 and 6.4 U) were used for inactivating PE. After 12 hours of incubation, MP at 3.2 U showed about 29% PE inactivation while MP at 6.4 U showed about 40% inactivation, 1.4 times higher than that of MP at 3.2 U. After incubating for four days, PE was inactivated approximately 76% for MP at 3.2 U and approximately 90% for MP 6.4 U. PE inactivation by MP at 6.4 U was 1.2 times faster than that by MP at 3.2 U. The decrease in PE activity from 12 to 96 hours for both MP at 3.2 U and MP at 6.4 U was 47 and 50%, respectively. There was no

Figure 2. Chromatography of mullet protease on Sephadex G-100. The column was eluted with 50 mM Tris-HCl at pH 7.8.

A 280 nm
Amidase activity
A 280 nm
Amidase activity (U/mL)
Fraction Number
0
0.5
1
1.5
2
2.5
3
3.5
0
5
10
15
20
25
1
11
21
31
41
51
61

Figure 3. Chromatography of mullet protease on Sephadex G-100 Superfine. The column was eluted with 50 mM Tris-HCl at pH 7.8.

A 280 nm
Amidase activity
A 280 nm
Amidase activity (U/mL)
Fraction Number
0
0.2
0.4
0.6
0.8
1
1.2
0
5
10
15
20
25
30
1
11
21
31
41
51
61

Figure 4. Effect of mullet protease (MP) on pectinesterase (PE) and thermostable pectinesterase (TSPE). PE was incubated with 3.2 and 6.4 units of MP and TSPE was incubated with 1.9 and 3.8 units of MP at room temperature.

Residual PE activity (%)
Time (Hour)
MP3.2
MP6.4
MP1.9
MP3.8
Control
0
20
40
60
80
100
120

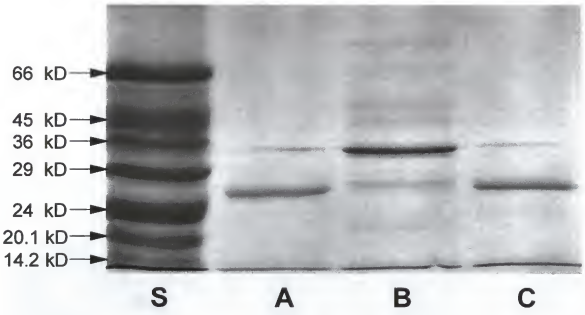
significant difference in the inactivation of PE from 12 to 96 hours between MP at 3.2 U and MP at 6.4 U.

Two different concentrations of MP (1.9 and 3.8 U) were used to inactivate TSPE (2.6 U). After 12 hours of incubation, MP at 1.9 U showed only 1% inactivation, while MP at 3.8 U showed about 16% inactivation, 16 times higher than that of MP at 1.9 U. After four days of incubation, TSPE inactivation by MP at 1.9 U was about 62% while that of MP at 3.8 U was approximately 91%. TSPE inactivation by MP at 3.8 U was 1.5 times higher than that by MP at 1.9 U. The decrease in TSPE activity from 12 to 96 hours for both MP at 1.9 U and MP at 3.8 U was 61 and 65%, respectively. There was no significant difference in inactivating TSPE from 12 to 96 hours between MP at 1.9 U and MP at 3.8 U.

Figure 5 shows the hydrolysis of PE by MP on a 12% SDS-PAGE gel. The intensity of the band near 36 KDa (Fig. 5. lane A) considered TLPE decreased significantly after four days of incubation. This demonstrates that PE was hydrolyzed by MP and inactivated.

Guizani (1991) reported that mullet protease separated by p-aminobenzamidine Sepharose 6B column and then a phenyl Sepharose 6B column inactivated pectinesterase. Mullet proteases from the pyloric ceca of mullet could inactivate other native enzymes such as pectinesterase. These proteases

Figure 5. Hydrolysis of pectinesterase by mullet protease on 12% SDS gel.(A) mullet protease; (B)pectinesterase; (C) a mixture of MP + PE; (S) standards.


66 kD
45 kD
36 kD
29 kD
24 kD
20.1 kD
14.2 kD
S
A
B
C

may be useful for the inactivation of target enzymes in food processing without any heat treatment.

PURIFICATION AND CHARACTERIZATION OF PROTEASES FROM THE HEPATOPANCREAS OF CRAWFISH (*Procambarus clarkii*) AND THEIR EFFECT ON PECTINESTERASES AND PECTATE LYASE C

Introduction

Enzymes have been used as processing aids in the manufacturing of food products. More specifically, proteases have been used in a number of industrial applications including laundry detergents, feed, leather-bating, silk degumming, cheese making, chill proofing, meat tenderizing, fermented sauces, and the production of pharmaceuticals (Simpson and Haard, 1987b). Recent interest has developed concerning proteases found in stomachless marine organisms which show no distinct stomach. Stomachless marine organisms are deprived of the acid denaturation which takes place in the stomach, making the protein in the feed more amenable to subsequent degradation in the intestine by trypsin, chymotrypsin and other proteases or peptidases. Trypsin and chymotrypsin from higher vertebrates do not hydrolyze native proteins or do so at a very low rate compared to denatured proteins (Jany, 1976; Mihalyi, 1978). Other evidence has

demonstrated that a protease from crayfish hydrolyzed native ribonuclease and inactivated native lactate dehydrogenase while proteases from organisms having a stomach were not effective (Pfleiderer et al., 1967). In this study it is hypothesized that the proteases from stomachless organisms could be important biotechnological molecules for food processing, especially inactivation of native deleterious enzymes in food systems. The objective of this work was to purify proteases from crawfish and to characterize these proteases as to their kinetic properties.

Materials and Methods

Louisiana swamp crawfish (*Procambarus clarkii*) was purchased live from a local seafood store. The crawfish was decapitated (Fig. 6) and the hepatopancreas removed, frozen rapidly in liquid nitrogen and then ground to a fine powder in a Waring blender. The hepatopancreas powder was stored at-70°C prior to extraction of the enzyme.

Extraction of Crawfish Protease

Twenty grams of crawfish hepatopancreas powder was mixed with 100 mL of ice-cold distilled water, 24 mL of tetrachloromethane, 70 mg of ascorbic acid and homogenized at

Figure 6. Crawfish showing the hepatopancreas organ.


Hepatopancreas

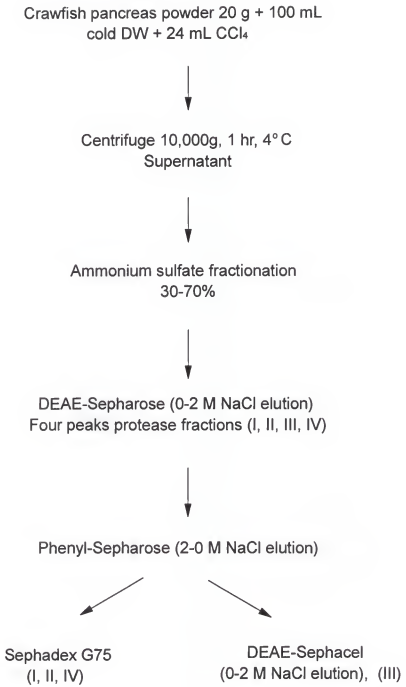
3 bursts for 30 seconds (total 90 seconds) at 5,000 rpm using a Polytron. The homogenate was centrifuged at 10,000g for one hour at 4°C. The supernatant was collected and referred to as defatted crude extract.

Chromatographic Procedures for Protease Purification

Figure 7 shows the purification protocol for purifying crawfish proteases. The defatted extract was fractionated with solid ammonium sulfate and the fraction sedimenting between 30 and 70% saturation was collected by centrifugation at 10,000g for one hour at 4°C. The precipitate from the ammonium sulfate fractionation was dissolved in 10 mM Tris-HCl buffer (pH 7.0).

The ammonium sulfate fraction was loaded onto a column of DEAE-Sepharose (2.5 cm id. x 30 cm). Elution was carried out with 10 mM Tris-HCl buffer, pH 7.0 at a rate of 0.2 mL/min with a linear gradient of 0 - 2 M NaCl. Material passing through the column was collected in 4 mL/tube fractions using a Bio-Rad fraction collector (Model 2110). The absorbance of the fractions was determined at 280 nm using a Beckman DU-7 spectrophotometer. Four distinct peaks showing esterase activity using tosylarginine methyl ester (TAME) as substrate were designated as CP-I, II, III, and IV in the order of their elution. These four fractions showing esterase activity were

Figure 7. Flow diagram for crawfish protease purification. DW-distilled water; CCl_4 - tetrachloromethane.


Crawfish pancreas powder 20 g + 100 mL
cold DW + 24 mL CCl_4
Centrifuge 10,000g, 1 hr, 4° C
Supernatant
Ammonium sulfate fractionation
30-70%
DEAE-Sepharose (0-2 M NaCl elution)
Four peaks protease fractions (I, II, III, IV)
Phenyl-Sepharose (2-0 M NaCl elution)
Sephadex G75
(I, II, IV)
DEAE-Sephacel
(0-2 M NaCl elution), (III)

pooled, concentrated using an Amicon cell (10 K), and loaded onto a Phenyl-Sepharose column (1.6 cm id. x 40 cm) which was equilibrated with 2 M NaCl in 10 mM Tris-HCl buffer, pH 7.0. Elution was carried out with an inverse linear gradient of 2 - 0 M NaCl in 10 mM Tris-HCl buffer, pH 7.0 at a rate of 0.2 mL/min. CP-I, II, and IV fractions showing esterase activity from the Phenyl-Sepharose column were pooled, concentrated and applied onto a column (1.0 cm id. X 40 cm) packed with Sephadex G-75. Elution was accomplished with a 10 mM Tris-HCl buffer, pH 7.0 at a rate of 0.13 mL/min. Material passing through the column was collected in 4 mL/tube fractions.

CP-III fractions showing esterase activity on a Phenyl-Sepharose column were pooled, concentrated and applied onto a DEAE-Sephacel column(1.0 cm id. X 50 cm). Elution was carried out with a linear gradient of 0 - 2 M NaCl in 10 mM Tris-HCl buffer, pH 7.0 at a rate of 0.2 mL/min. Material passing through the column was collected in 4 mL/tube fractions.

Protein Assay

Dye binding assay was used for quantitative determination of protein using bovine serum albumin (Fisher Scientific Company, Fair Lawn, NJ) as the standard. Protein assay

reagents were purchased from Bio-Rad Laboratories (Richmond, CA).

To estimate absorbance coefficient of proteins, absorbance of protein was measured at 280 nm (25°C) and divided by protein concentration (mg/mL).

Protease Activity Measurement

Esterase activity of the proteases was determined based on the method of Hummel (1959). Hydrolysis of tosylargine methyl ester (TAME) was measured by following the increase in absorbance at 247 nm in a DU-8 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). One hundred microliters of the enzyme solution was added to 0.3 mL of 10 mM TAME and 2.6 mL of 46 mM Tris-HCl, at pH 8.1 containing 11.5 mM $CaCl_2$ and the release of tosyl arginine was measured at 247 nm at 25°C. One unit of TAME activity is defined as ΔA247 nm/min x 1000 x 3 divided by 540, the molar absorption coefficient for tosyl arginine (Anonymous, 1988).

Electrophoresis and Molecular Weight Determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)was performed by the method of Laemmli (1970). For enzyme purity and molecular weight estimation of the crawfish proteases, separations were carried out on a Mini-Protean II

Dual Slab Cell (Bio-Rad Laboratories, Richmond, CA) using a 12% running gel with a 4% stacking gel. Proteins were diluted (1:4) with sample buffer (0.065 M tris-HCl buffer, pH 6.8, containing 10% glycerol, 1% SDS, 0.005% bromophenol blue and 1% 2-mercaptoethanol) and denatured by heating at 100°C for 4 min. Proteins were then loaded onto 1 mm thick gels and run in a buffer of 0.6% Tris, 2.9% glycine and 0.1% SDS, pH 8.3 with a current of 120 V. Following electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 (Eastman Kodak Co., Rochester, NY) in 40% ethanol and 10% acetic acid, and then destained in 40% ethanol and 10% acetic acid.

Molecular weights of the protein bands were determined by the method of Weber and Osborn (1969) using a low molecular weight standard kit (Sigma Chemical Co, Saint Louis, MO) containing: bovine serum albumin (66 kDa),egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase(rabbit muscle, 36 kDa), carbonic anhydrase (bovine erythrocytes, 29 kDa), trypsin (24 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Effect of pH on the Protease Activity

Activity of proteases was determined at different pHs using TAME as substrate at 25°C. The pH optimum for the hydrolysis of TAME was measured in various buffer solutions as

follows: 0.1 M citrate-HCl, pH 2.0; 0.1 M citrate-NaOH, pH 3.0; 0.1 M citrate-NaOH, pH 4.0; 0.1 M citrate-NaOH, pH 5.0; 0.1 M citrate-NaOH, pH 6.0; 0.1 M Tris-HCl, pH 7.0; 0.1 M Tris-HCl, pH 7.5; 0.1 M Tris-HCl, pH 8.0; 0.1 M Tris-HCl, pH 8.5; 0.1 M Tris-HCl, pH 9.0; and 0.1 M glycine-NaOH, pH 10.0.

pH Stability of the Proteases

The stability of the proteases at different pH values was determined by preincubating the enzyme with an equal volume of the various pH solutions described above for 30 min at 25°C prior to assaying for esterase activity with TAME (pH 8.1).

Effect of Temperature on the Protease Activity

The activity of the crawfish proteases was determined at different temperatures using TAME as substrate. After equilibrating an aliquot of the substrates at various temperatures (25-85°C), the protease was added and the initial linear activity was measured. The energy of activation (Ea) was then calculated from the data obtained at 5°C intervals from 25°C to 55°C using Arrhenius equation.

Temperature Stability of the Proteases

Thermostability of the four crawfish proteases was determined by measuring esterase activity at various

temperatures. The enzyme solution in 10 mL tube was incubated at the temperatures between 25°C to 60°C in a water bath for 5 minutes, removed immediately, cooled rapidly in an ice bath for 5 minutes, and added to the substrate solution at 25°C for determining residual esterase activity.

Isoelectric Focusing

Precast polyacrylamide isoelectric focusing gels (IEF Ready Gel, pH 3-10) and isoelectric focusing standards (Broad pI kit, pH 3.5-9.3) were purchased from Bio-Rad Laboratories (Richmond, CA) and Pharmacia (Piscataway, NJ), respectively. The purified proteases and standard proteins were applied onto the gel. The focusing was done at 100 V for an hour, 250 V for a hour and then 500 V for 30 min in a Mini-Protean II dual Slab Cell using 7 mM phosphoric acid as anode buffer and 20 mM lysine/20mM arginine as cathode buffer. Following focusing, the gel was stained with 0.05% Coomassie blue, 0.05% crocein scarlet in 27% ethanol and 10% acetic acid, and destained with a destaining solution of 40% methanol and 10% acetic acid.

Kinetic Studies

The hydrolysis of TAME was measured at various concentrations at 25°C. The kinetic constants (Km and Vmax) were determined by Lineweaver-Burk plots. The physiological

efficiency (Vmax/Km) was calculated as described by Pollock(1965).

Effect of Metal Ions on the Protease Activity

The effect of bivalent ions such as $CaCl_2$ (5 mM), $MgCl_2$ (5 mM), $ZnCl_2$ (1 and 5 mM), $CuCl_2$ (1 mM) and $HgCl_2$ (1 to 25 mM) on crawfish protease activity was determined. Each ion solution was incubated with equal amounts of enzyme solution at 25°C for 30 min before measuring the initial rate of the esterase activity with TAME as substrate.

Effect of Inhibitors on the Protease Activity

The effect of protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF, 5 mM), tosyl lysine chloromethyl ketone (TLCK, 2 mM), tosyl phenylalanine chloromethyl ketone (TPCK, 2 mM), aprotinin (0.2 TIU), soybean trypsin inhibitor (SBTI,5 µM), and ethylenediaminetetraacetate (EDTA, 5 mM) on the activity of crawfish proteases was determined.

Each inhibitor was incubated with an equal volume of enzyme solution at 25°C for 30 min and the initial rate of the reaction was measured at 247 nm using TAME (pH 8.1) as substrate.

Analysis of Amino Acid Composition

Purified crawfish proteases were hydrolyzed in 6N HCl for 24 hours in vacuum tubes at 120°C using PICO TAG work station (Waters, Milford, MA). Amino acids were resolved and quantified using an Applied Biosystem 420A Derivatizer (Applied Biosystems, Foster, CA) with amino acid standard from Pierce (Rockford, IL). Cystine and tryptophane were destroyed in hydrolysis, therefore not determined by this method. Amino acid analyses were carried out by R. Davenport at the Protein Chemistry Core facility, University of Florida.

Preparation of Antibody

Immunological cross reactivity between crawfish proteases was tested using CP-II as an antigen to induce antibody formation in chickens (50 week old commercial type egg laying white leghorn hens). Two chickens were injected at a wing (70 µg) and a foot pad (30 µg) with 100 µg of the enzyme in 1 mL distilled water and boosted after 2 weeks with an additional 100 µg. Antibodies were purified from egg yolk by the method of Polson et al. (1985). Yolk was separated from the white, diluted with 4 volumes of 0.1 M sodium phosphate buffer, pH 7.6 and mixed with 3.5% (w/v) polyethylene glycol (PEG) until homogeneous. The egg yolk suspension was centrifuged at 5,000g for 20 minutes, and the precipitate discarded. The supernatant

was thoroughly mixed with 8.5% (w/v) with PEG. After standing for 10 min, the mixture was centrifuged at 5,000g for 25 min. The precipitate obtained was dissolved in the 0.1 M phosphate buffer at a volume equal to 2.5 times that of the egg yolk. The mixture was made to 12% (w/v) with PEG and allowed to stand for 10 min before centrifugation at 5,000g for 25 min. The pellet was dissolved in 0.1 M phosphate buffer at a volume equal to 0.25 times that of the egg yolk, cooled to 0°C and an equal volume of 50% ethanol(v/v) precooled at -20°C was added to the mixture. After centrifugation at 10,000g for 25 min, the pellet obtained was dissolved in the 0.1 M phosphate buffer at a volume equal to 0.25 times that of the egg yolk. The antibody solution was dialyzed with stirring at 4°C against 50 volumes of the original 0.1 M phosphate buffer for 24 hours, and made to 0.1% sodium azide and stored at 4°C.

ELISA Procedures

Antigen was diluted to the appropriate concentration (1 µg/mL) into coating buffer (0.1 M sodium carbonate, pH 9.6) and 100 µL was added to each well of an Immulon plate (Fisher Scientific Company, Fair Lawn, NJ). After sitting for 2 hours at room temperature (or overnight at 4°C), the Immulon plate was washed four times with PBS-Tween buffer. One hundred microliters of antibody solution, diluted 1/1000, 1/10,000 and

1/100,000 with PBS-Tween was then added to each well. After 2 hours at room temperature (or overnight at 4°C), the plate was washed with PBS-Tween four times. Anti-chicken alkaline phosphatase conjugate (Sigma Chemical Co, Saint Louis, MO) was diluted 1/500 with PBS-Tween, and 75µL was added per well. After 2 hours at room temperature, the plate was washed four times with PBS-Tween. Alkaline phosphatase substrate was prepared by dissolving p-nitrophenol phosphate (Bio-Rad Laboratories, Richmond, CA) in substrate buffer (0.1 M sodium carbonate, 0.5 mM magnesium chloride, pH 9.4) at a concentration of 1 mg/mL and this was added to each well (75 µL per well). The absorbance at 405 nm was read with an ELISA reader (Bio-Rad). For blank determination, either the antigen or primary antibody was omitted; the higher color intensity of the two is taken as the blank determination.

For competitive ELISA experiments, various amounts of competitors (20-80 ng) were mixed with the primary antibody solution prior to allowing it to interact with the bound antigen. As antigen, 100 ng of CP-II was plated and anti-CP-II antibody was used at 1/10 dilution. Either CP-I, II, III, IV or pepsin, 20-80 ng, were added as competitors to the anti-CP-II antibody. To exclude the possibility of the antibody being digested by the protease, benzamidine (10 mM)

was added to the protease solution prior to incubation with the antibody.

Pectinesterase Assay

Pectinesterase (PE) activity was measured titrimetrically using a 1% pectin solution as substrate. The 1% pectin solution was prepared by adding pectin slowly to a 0.1 M sodium chloride solution while stirring at 40°C. After cooling to room temperature, sodium azide was added to a 1 mM final concentration.

This solution was adjusted to pH 7.5 at 25°C. While stirring 25 mL of the pectin solution, the PE sample was added and the initial reaction velocity was measured by automatic titration of the liberated carboxyl groups with standardized 0.01 N sodium hydroxide in a Combi recording pH-stat (Metrohm Ltd., Herissau, Switzerland). One unit of PE activity is defined as the amount of carboxyl groups liberated per minute at standard assay conditions.

Effect of Crawfish Proteases on Orange Pectinesterase

Ten units of commercial orange peel PE (Sigma Chemical Co, Saint Louis, MO) were mixed with twenty units of crawfish proteases and incubated at room temperature. Pectinesterase activity was measured with time by the titrimetric method.

Effect of Crawfish Proteases on Tomato Pectinesterase

Twelve units of the crawfish proteases were mixed with three units of tomato (*Lycopersicum esculentum*) pectinesterase provided by Dr. Greg Tucker at the University of Nottingham (Markovic and Jornvall, 1986) and incubated at room temperature. Pectinesterase activity was measured with time by the titrimetric method.

Effect of Crawfish Proteases on Pectate Lyase C

To study how crawfish proteases work on proteins, selective proteolysis of pectate lyase C was carried out following the protocol of Hurlbert (1996). Pectate lyase C (PLc) purified from *Erwinia chrysanthemi EC16* (Preston et al., 1992) was provided by Dr. J. Preston, Microbiology and Cell Science Department, University of Florida. In 30 µL reactions employing trypsin (Sigma Chemical Co, Saint Louis, MO) or crawfish proteases, 4 µg (20 µL of 200 µg/mL) of PLc was digested by 0.1 µg (5 µL of 20 µg/mL) of the proteases. Samples were removed at 0, 2, 8, 12, 18 and 24 hours and lyase activity was measured at 235 nm.

Lyase Activity Analysis of Protease Digested Pectate Lyase

For pectate lyase activity, samples (1 µL) were removed and added to 499 µL of buffer A (50 mM Tris-HCl, 0.2 mM CaCl2,

pH 8.0). Ten microliter aliquots of this solution were added to a one mililiter solution of 0.1% polygalacturonic acid (PGA) in buffer A. Activity was determined by measuring the change in absorbance at 235 nm on a Beckman DU-7 spectrophotometer.

MALDI-TOF Mass Spectrometry of Pectate Lyase Proteolysis

For Matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometer analysis, 2 µL of the digested lyase sample was removed and added to 2 µL of 0.1% triflouroacetic acid (TFA). One µL of matrix solution (saturated sinapinic acid in 40% CH_3CN/0.1% TFA) was added to 1 µL of sample solution to a sample pin and air dried. The sample was analyzed on a Vestec Research H Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometer employing a nitrogen laser to provide desorption energy. Data were analyzed with GRAMS/386 software package (Galactic Industries) running on a Zeos 486/33 mHz computer.

Results and Discussion

Purification of Crawfish Proteases

After ammonium sulfate fractionation (30-70%) and dialysis, crude defatted crawfish proteases were applied to a DEAE-Sepharose column. At between 0.3-1.2 M sodium chloride, significant protein peaks lacking esterase activity were eluted. Four distinct esterase activity peaks were then eluted (Fig. 8) and these were designated as CP-I, II, III and IV in the order of elution. These fractions were eluted at between 1.2-2.0 M sodium chloride; CP-I (1.19-1.32 M), CP-II (1.33-1.51 M), CP-III (1.52-1.70 M) and CP-IV (1.91-2.00 M). Elution of these enzymes at high salt concentration indicates that the crawfish proteases possess a high negative charge density on the molecular surface at the pH employed. For each activity peak, fractions were pooled and concentrated, and then applied to a Phenyl Sepharose column. Hydrophobic interaction chromatography was carried out by an inverse linear gradient of 2.0-0.0 M sodium chloride in 10 mM Tris-HCl, pH 7.0 on the Phenyl Sepharose column. One peak showing esterase activity was observed at between 0.82-0.62 M sodium chloride from the Phenyl Sepharose chromatography of CP-I (Fig.9). These

Figure 8. Chromatography of crawfish proteases using DEAE-Sepharose. The column was eluted with a 0-2 M NaCl linear gradient in 10 mM Tris-HCl at pH 7.0. Four activity peaks were designated as CP-I, CP-II, CP-III and CP-IV in the order of elution.

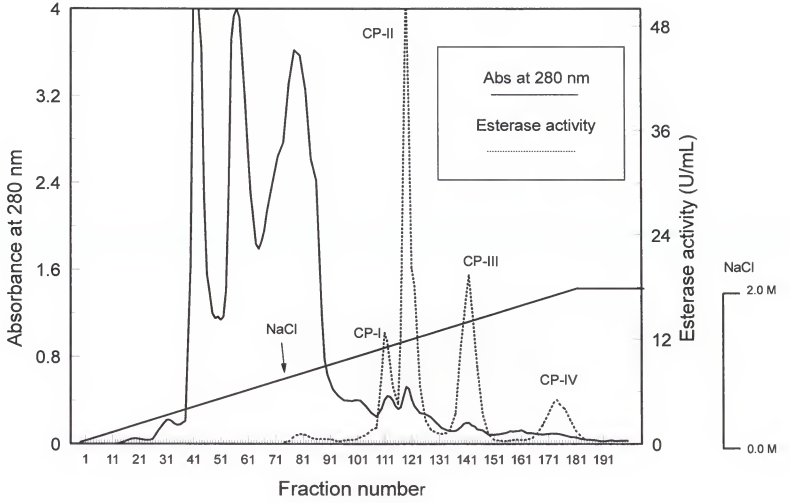
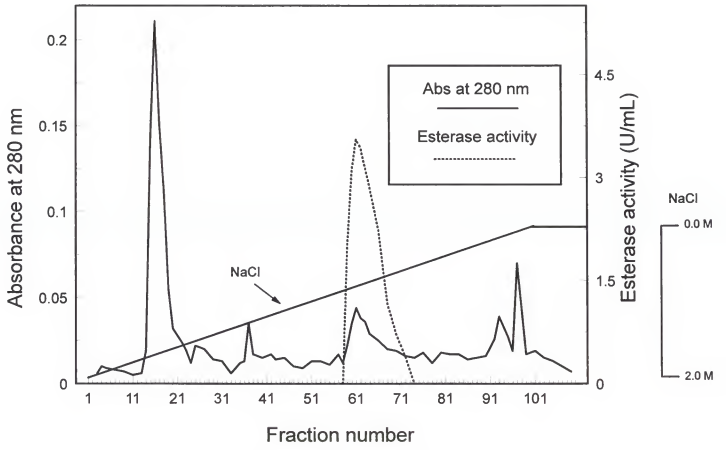

Absorbance at 280 nm
Esterase activity (U/mL)
Fraction number
Abs at 280 nm
Esterase activity
CP-I
CP-II
CP-III
CP-IV
NaCl
NaCl
2.0 M
0.0 M
0
0.8
1.6
2.4
3.2
4
0
12
24
36
48
1 11 21 31 41 51 61 71 81 91 101 111 121 131 141 151 161 171 181 191

Figure 9. Chromatography of CP-I using a Phenyl-Sepharose column. The column was eluted with an inverse linear gradient (2-0 M NaCl) in 10 mM Tris-HCl at pH 7.0.


Absorbance at 280 nm
0.2
0.15
0.1
0.05
0
Abs at 280 nm
Esterase activity
NaCl
4.5
3
1.5
0
Esterase activity (U/mL)
NaCl
0.0 M
2.0 M
1
11
21
31
41
51
61
71
81
91
101
Fraction number

Sephadex G-75 column, and one esterase activity peak was observed (Fig. 10).

When CP-II was applied to the Phenyl Sepharose column one peak showing esterase activity was observed at 0.0 M sodium chloride (Fig. 11). These fractions (59-68) were pooled, concentrated and applied to the Sephadex G-75 column (Fig. 12) and one esterase activity peak resulted.

Finally, one CP-III peak showing esterase activity was observed at 0.0 M sodium chloride on the Phenyl Sepharose column (Fig. 13). These fractions (97-106) were pooled, concentrated and applied to a DEAE-Sephacel column (Fig. 14) using a linear gradient of 0.0 - 2.0 M sodium chloride in Tris-HCl, pH 7.0. Fractions (41-50) showing esterase activity were observed at between 0.96-1.18 M sodium chloride.

One CP-IV peak showing esterase activity was observed at 0 M sodium chloride on the Phenyl Sepharose column using an inverse linear gradient (Fig. 15). These fractions (102-108) were pooled, concentrated and applied to a Sephadex G-75 column (Fig. 16) and one esterase activity peak was found.

After all the chromatography steps were performed, four crawfish proteases were observed to migrate as a single band when run on 12% SDS-PAGE(Fig. 17).

Results from the purification of the four crawfish proteases based on esterase activity for TAME are summarized

Figure 10. Chromatography of CP-I on a Sephadex G-75 column. The column was eluted with 10 mM Tris-HCl at pH 7.0.

Absorbance at 280 nm
0.12
0.09
0.06
0.03
0
Esterase activity (U/mL)
12
9
6
3
0
1
6
11
16
21
26
31
36
41
46
Fraction number
Abs at 280 nm
Esterase activity

Figure 11. Chromatography of CP-II using a Phenyl-Sepharose column. The column was eluted with an inverse linear gradient (2-0 M NaCl) in 10 mM Tris-HCl at pH 7.0.

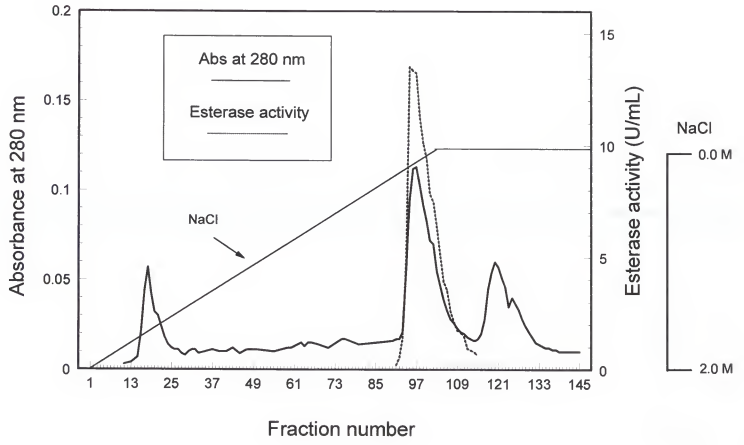

Abs at 280 nm
Esterase activity
NaCl
Absorbance at 280 nm
Esterase activity (U/mL)
Fraction number
0.2
0.15
0.1
0.05
0
15
10
5
0
1
13
25
37
49
61
73
85
97
109
121
133
145
NaCl
0.0 M
2.0 M

Figure 12. Chromatography of CP-II on a Sephadex G-75 column. The column was eluted with 10 mM Tris-HCl at pH 7.0.

Absorbance at 280 nm
0.45
0.3
0.15
0
1
6
11
16
21
26
31
36
41
Fraction number
Esterase activity (U/mL)
40
30
20
10
0
Abs at 280 nm
Esterase activity

Figure 13. Chromatography of CP-III using a Phenyl-Sepharose column. The column was eluted with an inverse linear gradient (2-0 M NaCl) in 10 mM Tris-HCl at pH 7.0.

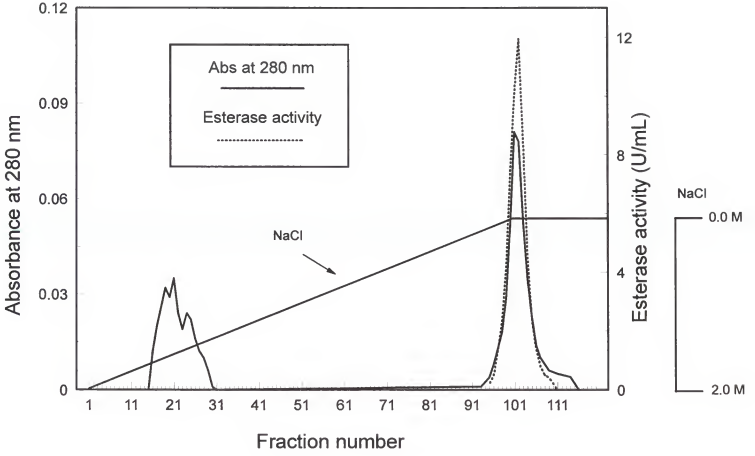

Abs at 280 nm
Esterase activity
NaCl
Absorbance at 280 nm
Esterase activity (U/mL)
Fraction number
0.12
0.09
0.06
0.03
0
12
8
4
0
1
11
21
31
41
51
61
71
81
91
101
111
NaCl
0.0 M
2.0 M

Figure 14. Chromatography of CP-III on a DEAE-Sephacel column. The column was eluted with a linear gradient (0-2 M NaCl) in 10 mM Tris-HCl at pH 7.0.

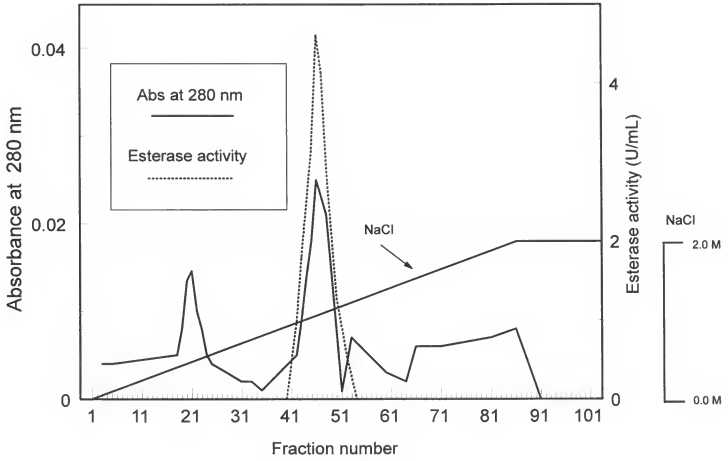

0.04
0.02
0
Absorbance at 280 nm
Abs at 280 nm
Esterase activity
NaCl
4
2
0
Esterase activity (U/mL)
NaCl
2.0 M
0.0 M
1
11
21
31
41
51
61
71
81
91
101
Fraction number

Figure 15. Chromatography of CP-IV using a Phenyl-Sepharose column. The column was eluted with an inverse linear gradient (2-0 M NaCl) in 10 mM Tris-HCl at pH 7.0.

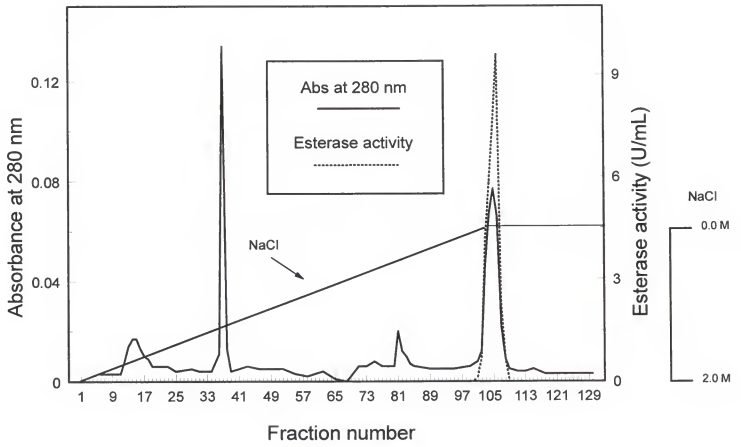
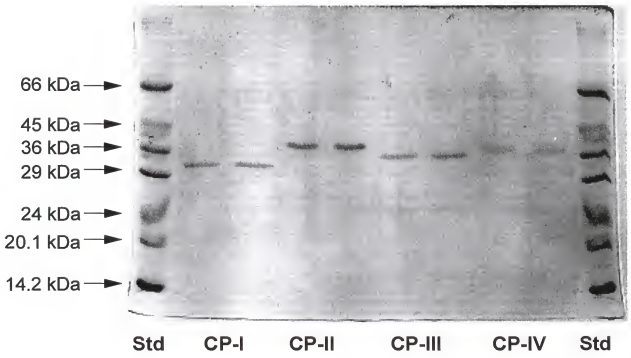

Abs at 280 nm
Esterase activity
NaCl
Absorbance at 280 nm
Esterase activity (U/mL)
0.12
0.08
0.04
0
9
6
3
0
1 9 17 25 33 41 49 57 65 73 81 89 97 105 113 121 129
Fraction number
NaCl
0.0 M
2.0 M

Figure 16. Chromatography of CP-IV on a Sephadex G-75 column. The column was eluted with 10 mM Tris-HCl at pH 7.0.

Absorbance at 280 nm
0.09
0.06
0.03
0
1
6
11
16
21
26
31
36
41
Fraction number
Esterase activity (U/mL)
6
4
2
0
Abs at 280 nm
Esterase activity

Figure 17. SDS-PAGE of crawfish proteases following all chromatography steps. The purified crawfish proteases (CP I-IV) were applied onto a 12% SDS-PAGE gel. Each protease was applied to two lanes of the gel.


66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20.1 kDa
14.2 kDa
Std
CP-I
CP-II
CP-III
CP-IV
Std

in Table 3. After Sephadex G-75 gel filtration, purification for CP-I, CP-II and CP-IV increased to 48-, 70-, and 69- fold, respectively. After DEAE-Sephacel chromatography, purification of CP-III increased 71-fold. Having started with twenty grams of crawfish hepatopancreas, 160 µg of CP-I, 270 µg of CP-II, 60 µg of CP-III, and 87 µg of CP-IV were obtained. The yield for CP-II was higher (12.9%) than CP-I (1.9%) and CP-IV(4.2%) after Sephadex G-75 chromatography, while CP-II was also higher than CP-III (1.0%) after DEAE-Sephacel chromatography.

Absorbance Coefficients of the Crawfish Protease

Ultraviolet light absorption methods have several advantages:(1) they can be performed directly on the sample without the addition of any reagents, (2) they can be performed very rapidly since no incubations are required, and (3) the relationship between protein concentration and absorbance is linear (Stoscheck, 1990). It is not thought that Tris buffer affects the absorbance of protein at 280 nm since the concentration of Tris buffer (10 mM) for crawfish proteases was much lower than the concentration limit of Tris buffer (0.5 M) for measuring absorbance of protein at 280 nm. Molar extinction coefficients and absorbance coefficients for the crawfish proteases are found in Table 4. The molar extinction coefficient of CP-III was the lowest among all four

Table 3. Purification scheme for crawfish proteases

Step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Yield (%)	Purification fold
Crude Extract[b] (10,000g)	414	2,090	5.2	100.0	1.0
30-70% $(NH_4)_2SO_4$	269	1,767	6.6	84.5	1.3
DEAE-Sepharose					
CP - I	5.4	216.1	40.2	10.3	8.0
CP - II	5.4	533.9	98.2	25.5	19.4
CP - III	2.1	273.0	128.8	13.1	25.5
CP - IV	0.7	171.7	235.2	8.2	46.6
Phenyl-Sepharose					
CP - I	0.3	56.8	202.9	2.7	40.2
CP - II	1.2	328.0	282.7	15.7	56.0
CP - III	0.5	57.0	301.9	7.5	59.8
CP - IV	0.5	110.3	220.6	5.3	43.7
Sephadex G-75					
CP - I	0.2	39.1	244.5	1.9	48.4
CP - II	0.8	270.5	355.9	12.9	70.5
CP - IV	0.3	87.5	350.3	4.2	69.4
DEAE-Sephacel					
CP - III	0.1	21.6	360.5	1.0	71.4

[a] One unit catalyzes the hydrolysis of one µmol TAME/min at 25° C, pH 8.1
[b] Twenty grams of hepatopancreas was extracted.

enzymes. Crawfish hepatopancreas proteases showed much higher molar extinction coefficients compared with trypsins from *Stereptomyces griseus* (39,600) (Olafson and Smillie, 1975) and from carp pancreas (56,200) (Cohen et al., 1981).

The absorbance coefficients were useful to estimate the protein concentration by absorbance of proteins at 280 nm.

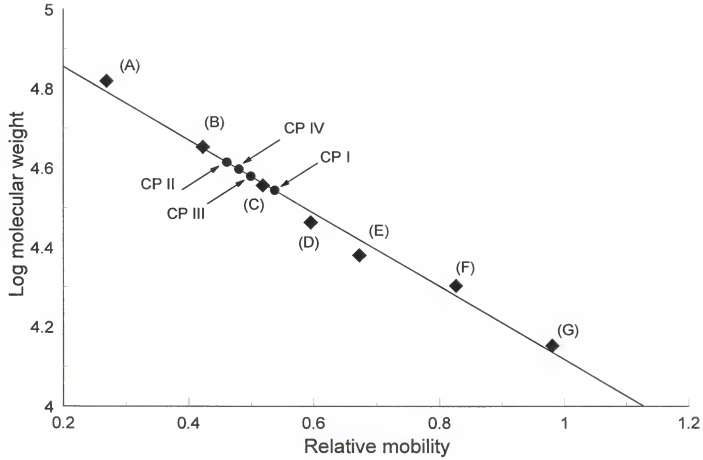
Table 4. Absorbance coefficients and extinction coefficients of crawfish proteases

Enzyme	$A_{1cm}^{1mg/ml}$	E_M
CP-I	3.86	135,100
CP-II	3.23	133,016
CP-III	2.27	86,033
CP-IV	3.58	141,410

Molecular Weights of Crawfish Proteases

The molecular weights for CP-I, CP-II, CP-III and CP-IV were determined to be 35.0, 41.2, 37.9 and 39.5 kDa, respectively, using SDS-PAGE (Figs. 17 and 18). Trypsin enzymes have been reported to have a molecular weight between 20 and 25 kDa (Keil, 1971). However, those from marine organisms, in most instances, have molecular weights in the range of 24-30 kDa. The apparent molecular weights for these crawfish proteases were higher than those reported for most crustacean trypsin-like proteases. The molecular weights of trypsin-like enzymes from many crustaceans have been reported

Figure 18. Molecular weight determination on SDS-PAGE for the purified crawfish proteases. The protein standards are (A) bovine serum albumin (66 kDa), (B) egg albumin (45 kDa),(C) glyceraldehyde-3-phosphate dehydrogenase (36 kDa), (D) carbonic anhydrase (29 kDa), (E), bovine trypsin (24 kDa), (F) soybean trypsin inhibitor (20.1 kDa), and (G) α-lactalbumin (14.2 kDa).

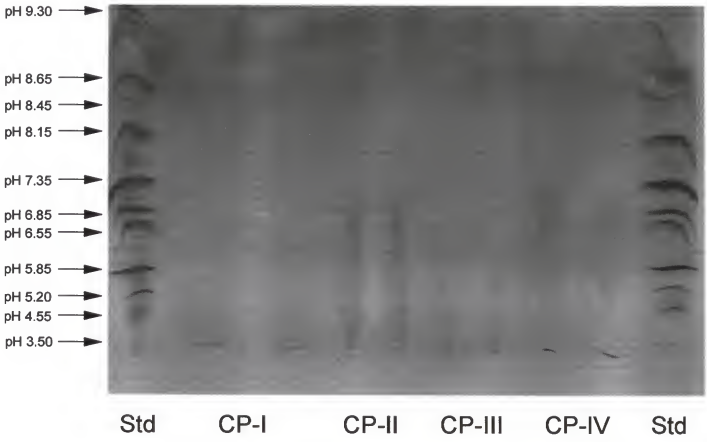

5
4.8
4.6
4.4
4.2
4
Log molecular weight
0.2
0.4
0.6
0.8
1
1.2
Relative mobility
(A)
(B)
(C)
(D)
(E)
(F)
(G)
CP I
CP II
CP III
CP IV

to be between 20-25 kDa: 24 kDa from white shrimp (*Penaeus setiferus*)(Gates and Travis, 1969), 20.5 kDa from rock crab (Brun and Wojtowicz, 1976), 24 kDa from Japanese spiny lobster (Galgani and Nagayama, 1987a), and 24 kDa from crayfish (DeVillez and Johnson, 1968). Some reports found trypsins of higher molecular weights: 33.5 kDa from the midgut gland of crab, *Callinectes sapidus* (Dendinger and O'Connor, 1990), 30-31 kDa from Antarctic krill (Osnes and Mohr, 1985), and 28-30 kDa from krill (Kimoto et al., 1983).

Isoelectric focusing and pI Determiation

Crawfish proteases showed isoelectric points around or less than 3.5 when focused on a polyacrylamide gel(pH 3-10) with isoelectric focusing standards (pH 3.5-9.3) (Fig. 19). This indicates that crawfish proteases are strongly anionic and high in acidic amino acids. Isoelectric points for the crawfish proteases are in agreement with other trypsin like enzymes reported for crustaceans. Isoelectric points for trypsin-like enzymes from crustacean digestive systems have been reported to be very low, i.e. 4.0 for the Atlantic blue crab, *Callinecters sapidus* (Dendinger and O'Connor, 1990), 3.8 for crayfish, *Astacus leptodactrylus* (Zwilling et al., 1969), 2.6 for Arctic krill (Kimoto et al., 1983) and less than 3.5 for Antarctic krill (Osnes and Mohr, 1985). In marine

Figure 19. Isoelectric focusing of crawfish proteases. The purified crawfish proteases (CP I-IV) and isoelectric focusing standards (pH 3.5-9.3) were applied onto a precast polyacrylamide isoelectric focusing gel (pH 3-10). Each protease was applied to two lanes of the gel.


pH 9.30
pH 8.65
pH 8.45
pH 8.15
pH 7.35
pH 6.85
pH 6.55
pH 5.85
pH 5.20
pH 4.55
pH 3.50
Std
CP-I
CP-II
CP-III
CP-IV
Std

organisms, anionic trypsins are found commonly in starfish (Winter and Neurath, 1970), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1984) and salmon (Pivnenko et al., 1990).

Effect of Temperature on the Proteases Activity

Increasing the temperature of an enzyme reaction causes the rate of the reaction to increase while also increasing its rate of inactivation (Stauffer, 1989). As a rule of thumb, an increase of 10°C will cause the reaction rate to double, while the inactivation rate will increase 64-fold.

The effect of temperature was examined by measuring esterase activity at various temperatures and the results are presented in Figure 20. The optimum temperature for the hydrolysis of TAME was 65°C for CP-II and 70°C for CP-I, CP-III and CP-IV. These temperatures were higher than those reported for proteases from Greenland cod (Simpson and Haard, 1984a) and caplin (Hjelmeland and Raa, 1982).

Activation Energy

The activation energy of a reaction is the amount of energy in calories required to bring all the molecules in 1 mol of a substance at a given temperature to the transition state at the top of the energy barrier (Lehninger, 1982).

Figure 20. Effect of temperature on the activity of the four crawfish proteases.

Relative activity (%)
120
100
80
60
40
20
0
25
30
35
40
45
50
55
60
65
70
75
80
85
Temperature (°C)
CP-I CP-II CP-III CP-IV

Thermodynamic free energy of activation (Ea) values for crawfish proteases' hydrolysis of TAME are summarized in Table 5. Crawfish proteases had activation energies around 7.0-8.3 kcal/mole, which are considerably lower than 13.4 kcal/mole for bovine trypsin (Simpson and Haard, 1984b).

Table 5. Activation energy of crawfish proteases

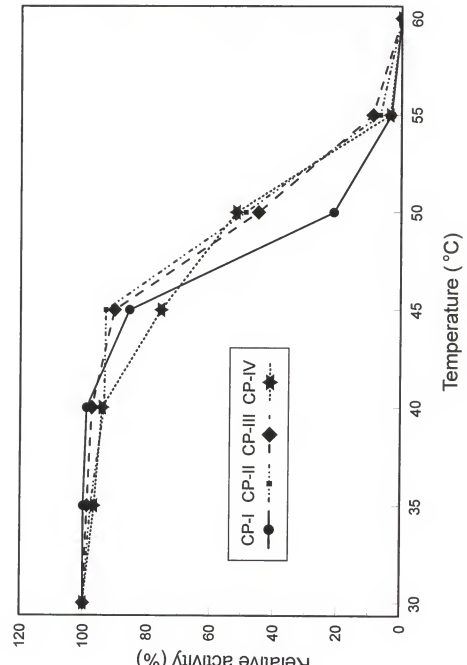
Enzyme	Ea (kcal/mol)
CP-I	8.34
CP-II	7.45
CP-III	7.07
CP-IV	6.98
Bovine trypsin*	13.4

* Data from Simpson and Haard (1984b).

Temperature Stability

Figure 21 shows the residual esterase activities of the proteases measured with 1 mM TAME at 25°C after 5 minutes incubation in a water bath at various temperatures. Heat treatment up to 40°C for 5 min did not affect esterase activity for CP-I and CP-IV, while 45°C for 5 min did not affect CP-II and CP-III. At 50°C, CP-II, III and IV showed approximately a 50% reduction in activity while CP-I showed approximately an 80% reduction in activity. All four crawfish proteases were nearly completely inactivated at 55°C.

Figure 21. Thermostability of the purified crawfish proteases. The enzymes were incubated for 5 min at various temperatures.


Relative activity (%)
120
100
80
60
40
20
0
30
35
40
45
50
55
60
Temperature (°C)
CP-I
CP-II
CP-III
CP-IV

Effect of pH on the Protease Activity

The effect of pH on esterase activity using TAME was examined and the results are presented in Figure 22. Esterase activity of all four crawfish proteases were low at acidic pH. The optimum pH for hydolysis of TAME at 25°C was 8.0. At pH 8.5, the activity remained at 90-99% of the pH 8.0 activity, however it decreased significantly to 20-33% as the pH approached 9.0 and no activity occurred at pH 10.0. Similar pH optima were reported for trypsin-like enzymes from invertebrates (Gates and Travis, 1969; Jany, 1976; Hjelmeland and Raa, 1982; Simpson and Haard, 1984a) and vertebrates (Erlanger et al., 1961).

pH Stability of the Protease Activity

After thirty minutes of preincubating the enzymes with various pH solutions, the esterase activity was measured and the results are presented in Figure 23. Crawfish proteases were stable at around pH 7.0-10. However, crawfish proteases were very unstable at acidic conditions. A similar inactivation at acid pH was shown to occur in anionic trypsins from crayfish (Zwilling et al., 1969), shrimp (Gates and Travis, 1969), crab (Muramatsu and Morita, 1981), capelin (Hjelmeland and Raa, 1982), atlantic blue crab (Dendinger and O'Connor, 1990) and sardine (Murakami and Noda, 1981).

Figure 22. Effect of pH on the activity of the four crawfish proteases.

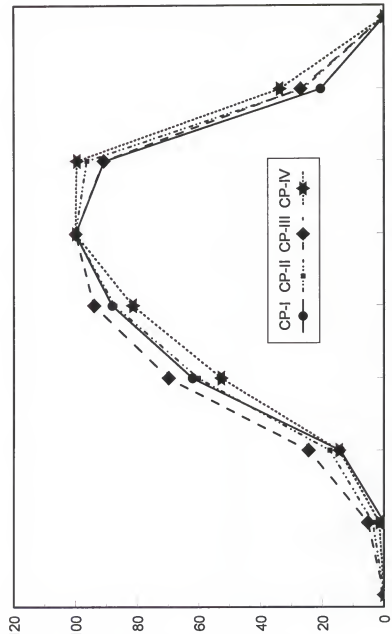
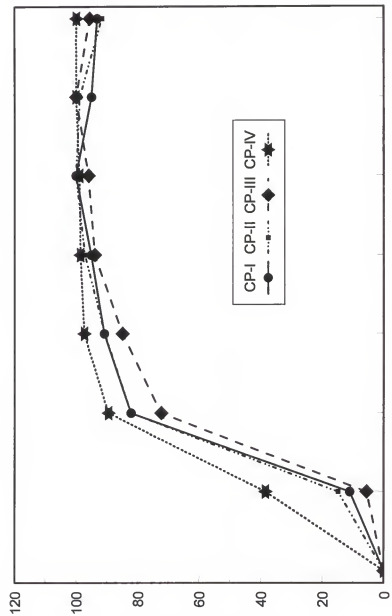

Relative activity (%)
120
100
80
60
40
20
0
4
5
6
7
7.5
8
8.5
9
10
pH
CP-I
CP-II
CP-III
CP-IV

Figure 23. pH stability of the four crawfish proteases. Enzymes were incubated with equal volumes of various pH buffers for 30 min at 25°C.


Relative activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH
CP-I CP-II CP-III CP-IV

Kinetic Constants

Kinetic constants, Km and Vmax were estimated by measuring esterase activity using TAME as substrate at different substrate concentrations. The Km and Vmax values were calculated using Lineweaver-Burk plots and are presented in Table 6. Lineweaver-Burk plot of the hydrolysis of TAME by CP-I is shown in Figure 24.

The Km value of CP-III was higher than the other three crawfish proteases. The turnover number of CP-I was lower than the other crawfish proteases. CP-I would be the least efficient catalyst in hydrolyzing TAME among the four enzymes.

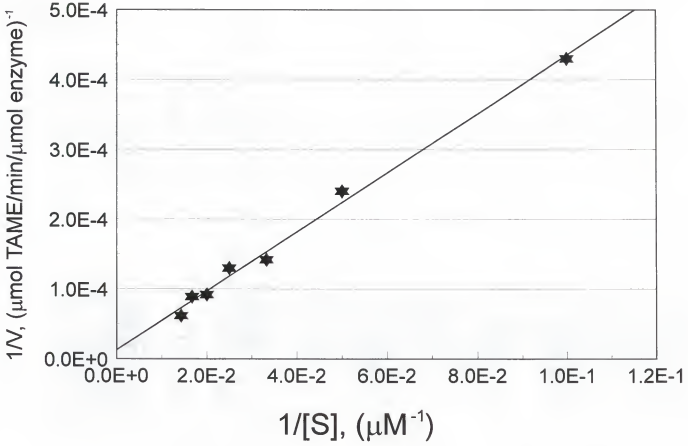
Table 6. Kinetic constants for the four crawfish proteases

Enzyme	Km (μM)	V_{max}* (min^{-1})	V_{max}/Km (μM^{-1} min^{-1})
CP-I	37.4	9,108	243.5
CP-II	35.7	15,444	432.6
CP-III	43.2	13,374	309.6
CP-IV	34.9	13,536	387.9

* Turnover number is TAME units per μM enzyme.

Physiological efficiency was estimated as the ratio of its sbstrate turnover number (Vmax) to its Michaelis constant (Km) (Pollock, 1965).

Figure 24. Lineweaver-Burk plot of the hydrolysis of TAME by crawfish protease CP-I. TAME concentration ranged from 10 to 70 μM.


5.0E-4
4.0E-4
3.0E-4
2.0E-4
1.0E-4
0.0E+0
1/V, (µmol TAME/min/µmol enzyme)-1
0.0E+0
2.0E-2
4.0E-2
6.0E-2
8.0E-2
1.0E-1
1.2E-1
1/[S], (µM-1)

The physiological efficiency value of CP-II was higher than those of other crawfish enzymes. This suggests that CP-II would be more efficient at transforming the substrate to product.

Effect of Metal Ions on the Protease Activity

The effect of metal ions on crawfish proteases activity is summarized in Table 7. Crawfish proteases were not affected significantly by calcium and magnesium ions but were affected by cupric and zinc ions. Figure 25 shows the effect of mercuric ions on the activity of the four crawfish proteases. Unlike other serine proteases, the activity of CP-I and CP-II were activated by mercury while CP-III and IV were inhibited. The activity of CP-I increased to 221% after 30 minutes of incubation with an 11 mM mercury ion solution while CP-II increased to 130% with a 5 mM mercury solution. Usually mercury inhibits the activity of serine proteases by modifying sulfhydryl groups of on the enzyme and therefore denaturing the protein. The possible explanation of activation by mercury might be that mercury modifies sulfhydryl groups which may modify the structure at the active site making it catalytically more efficient. To confirm this hypothesis, iodoacetamide, which specifically modifies sulfhydryl group on enzymes, should be examined in future studies.

Figure 25. Effect of mercury on the activity of the four crawfish proteases.

Relative activity (%)
250
200
150
100
50
0
0
5
10
15
20
25
Concentration (mM)
CP-I
CP-II
CP-III
CP-IV

Table 7. Effect of metal ions on crawfish proteases*

Metal ion	Conc. (mM)	Relative activity (%) CP-I	CP-II	CP-III	CP-IV
$CaCl_2$	5	100±3	98±4	95±5	99±4
$MgCl_2$	5	93±4	97±6	95±6	100±6
$CuCl_2$	1	30±5	33±4	24±5	42±6
$ZnCl_2$	1	77±6	76±4	81±7	65±4
$ZnCl_2$	5	42±4	37±5	46±5	20±3
$HgCl_2$	1	120±5	106±6	97±6	80±6
$HgCl_2$	5	160±5	130±6	45±5	35±6

* Metal ions were incubated with the enzyme for 30 minutes at 25°C before assaying.

Effect of Inhibitors on the Protease Activity

Serine protease inhibitors such as PMSF, TLCK, TPCK, aprotinin, EDTA and SBTI were examined on the activity of the four crawfish proteases (Table 8). All four crawfish proteases were inhibited by PMSF, TLCK, aprotinin, EDTA, SBTI but not by TPCK.

Phenyl methyl sulfonyl fluoride (PMSF) inhibited activities of all four crawfish proteases. PMSF has been described as a serine protease inhibitor which reacts with the essential serine at the active center of mammalian proteinases (Gold and Fahrney, 1964). PMSF inhibition of trypsins from

Table 8. Effect of protease inhibitors on crawfish proteases[a]

Inhibitor	Conc.	Inhibition (%)			
		CP-I	CP-II	CP-III	CP-IV
PMSF	5 mM	33.5	41.4	62.9	42.0
Aprotinin	0.2 TIU[b]	100.0	100.0	100.0	100.0
EDTA	5 mM	23.8	40.0	21.8	22.0
TLCK	2 mM	100.0	100.0	100.0	100.0
TPCK	2 mM	0.0	0.0	0.0	0.0
SBTI	5 µM	100.0	100.0	100.0	100.0

[a] Inhibitors were incubated with equal volumes of enzyme for 30 minutes at 25°C before assaying.
[b] TIU stands for trypsin inhibitor units.

bonefish (Jany, 1976) and capelin (Hjelmeland and Raa, 1982) has been reported.

Tosyl lysine chloromethyl ketone (TLCK) trypsin inhibitor inactivated completely all four crawfish proteases. TLCK inactivates only trypsin-like enzymes by forming a covalent bond with histidine at the catalytic portion of the molecule and then blocking the substrate-binding portion at the active center (Severin and Tomasek, 1965). Soybean trypsin inhibitor (SBTI) and aprotinin inhibited all four crawfish proteases completely. Crawfish proteases were 22-40% inhibited by EDTA. Trypsins from crustaceans such as lobster (Galgani and Nagayama, 1987a) and shrimp (Galgani and Nagayama,1987b) were inhibited approximately 20-35% by EDTA. Two anchovy trypsins were inhibited 34 and 60% (Martinez et al., 1988) while starfish trypsin was only slightly inhibited by EDTA (Winter and Neurath, 1970).

None of the crawfish proteases were inhibited by tosyl phenylalaine chloromethyl ketone (TPCK), a specific inhibitor for chymotrypsin.

These results suggest that crawfish proteases could be considered as serine-type proteases and classified as trypsin-like, lacking chymotrypsin activity.

Analysis of Amino Acid Composition

Crawfish proteases were rich in acidic amino acids, glycine and alanine but low in basic amino acids and methionine (Table 9).

The ratios of acidic to basic amino acids were 3.8 for CP-I, 4.1 for CP-II, 3.7 for CP-III, and 3.5 for CP-IV. These ratios were similar to 4.2 for shrimp (Gates and Travis, 1969), 4.3 for crayfish (Zwilling et al., 1969) but higher than 1.9 for bovine trypsin(Walsh and Neurath, 1964). The high ratio of acidic to basic amino acids supports the low isoelectric points (pI) for these proteases as well as the strong binding of these to DEAE-Sepharose, an anionic exchange resin.

Cysteine was destroyed during hydrolysis before amino acid analysis, and therefore was not determined in this study.

Antibody Preparation and Immunological Analysis

The titer of anti-CP-II chicken antibodies from eggs was determined by ELISA. One of two chickens injected with CP-II showed antibody activity around day 17 from the initial injection. Three eggs with highest titer (days 15,17, and 19 from the initial injection) were pooled and antibody against CP-II was purified. Immunological cross reactivity was analyzed by competitive ELISA between the crawfish proteases.

Table 9. Amino acid composition of crawfish proteases[a]

Amino Acid	mol %			
	CP-I	CP-II	CP-III	CP-IV
asx	12.72	12.33	13.66	11.64
glx	7.95	10.89	9.23	10.94
ser	5.47	3.71	6.04	4.05
gly	12.93	13.76	13.30	13.80
his	2.50	1.97	3.12	3.34
arg	1.22	1.22	0.94	0.56
thr	6.70	5.47	7.04	6.96
ala	10.05	8.60	9.67	8.06
pro	6.60	6.03	6.03	6.33
tyr	4.21	5.05	4.26	4.67
val	8.24	7.93	7.62	7.99
met	0.49	0.66	0.30	0.81
ile	6.53	6.70	6.26	7.31
leu	8.75	9.17	6.88	7.58
phe	3.86	4.02	3.48	3.37
lys	1.80	2.49	2.17	2.59

[a] Cystein and tryptophane were destroyed during the acid hydrolysis.

In the assay, CP-I, CP-II, CP-III, CP-IV and pepsin were used as competitors for anti-CP-II antibodies against plated antigen, CP-II. As shown in Figure 26, color intensity was reduced with increasing competitor concentration. When 80 ng for any CP enzyme was used, the color intensity (absorbance at 405 nm) was similar for all proteases. When pepsin was used as a competitor, no reduction in color intensity was observed. These results suggest that the four crawfish enzymes are cross reactive and, therefore, they have shared structural components.

Effect of Crawfish Proteases on Orange Pectinesterase

Inactivation of a commercial orange peel pectinesterase by crawfish proteases was examined. The results are shown in Table 10. In the inactivation of orange pectinesterase, CP-IV was the most effective followed by CP-II. Eventually PE was completely inactivated by CP-IV at 133 hours, and by CP-I and CP-II at 180 hours. There is no significant synergistic effect of crawfish proteases in inactivating orange pectinesterase. The mixture of CP-II and CP-IV showed lower residual PE activity (3.8%) of all combinations at 133 hours. However, this was still not as effective as CP-IV alone.

Figure 26. Analysis of antigenic properties of purified crawfish proteases by competitive ELISA. CP-II (100 ng) was plated and reacted with an anti-CP-II plus 20-80 ng/well of CP-I, CP-II, CP-III, CP-IV or pepsin. The figure is for one trial and this is representative of two additional trials.

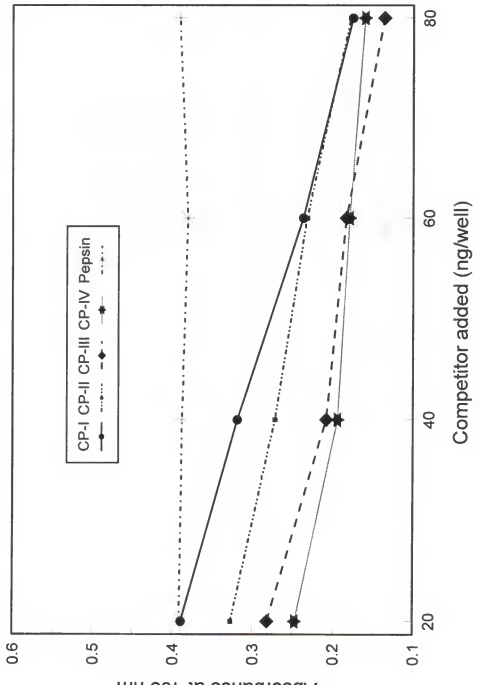

Absorbance at 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
Competitor added (ng/well)
CP-I CP-II CP-III CP-IV Pepsin

Table 10. Effect of crawfish proteases on orange pectinesterase[a]

Enzyme	Residual Pectinesterase Activity (%)					
	9 hr	14 hr	110 hr	133 hr	158 hr	180 hr
CP-I	100.0	87.8	43.2	18.3	9.4	0.0
CP-II	82.1	80.2	16.2	9.2	1.3	0.0
CP-III	99.7	96.3	60.8	45.1	42.3	20.8
CP-IV	88.3	79.3	19.2	0.0	0.0	0.0
CP-I+II[b]	93.1	86.7	28.6	15.3	6.2	0.0
CP-I+III[b]	100.0	93.2	54.1	30.2	24.8	12.1
CP-I+IV[b]	93.2	85.1	30.2	11.0	6.1	0.0
CP-II+III[b]	91.8	87.8	40.8	28.2	22.4	9.5
CP-II+IV[b]	86.2	79.0	18.2	3.8	0.0	0.0
CP-III+IV[b]	95.4	86.2	41.6	30.6	23.6	11.3

[a] Ten units of pectinesterase were incubated with twenty units of crawfish protease at room temperature.

[b] Crawfish proteases were mixed in the ratio of 1:1 (10 U: 10 U).

Effect of Crawfish Proteases on Tomato Pectinesterase

Inactivation of pectinesterase from tomato (*Lycopersicum esculentum*) by crawfish proteases was investigated and the results are shown in Figure 27.

In inactivating tomato pectinesterase, CP-II was the most effective followed by CP-IV. Tomato PE was totally inactivated by CP-II at 36 hours followed by CP-I and CP-IV at 48 hours, and then CP-III at 72 hours.

Effect of Crawfish Proteases on Pectate Lyase C

Figure 28 shows the time course of inactivation data by the four crawfish proteases and trypsin on Pectate lyase C (PLc) purified from *Erwinia chrysanthemi* EC16. The ratio (w/w) of protease to PLc was 1:40. After 8 hours digestion of PLc by the proteases, PLc was inactivated 12% by trypsin and about 20% by the crawfish proteases. After 18 hours of digestion, trypsin reduced PLc activity approximately 28% while crawfish proteases reduced the PLc activity around 40%. After 24 hours of digestion, about 63% lyase activity remained with trypsin and CP-II while 54% PLc activity remained with CP-III and CP-IV.

Figure 27. Effect of crawfish proteases on tomato pectinesterase. Three units of PE and twelve units of CP were incubated at room temperature. This figure is for one trial and this is representative of one additional trial.

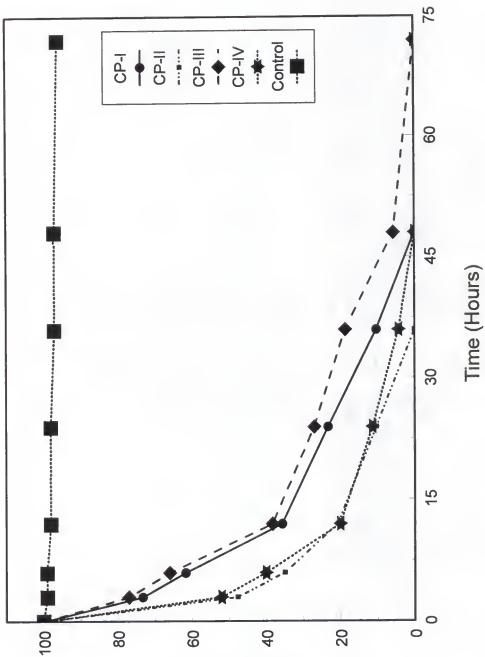
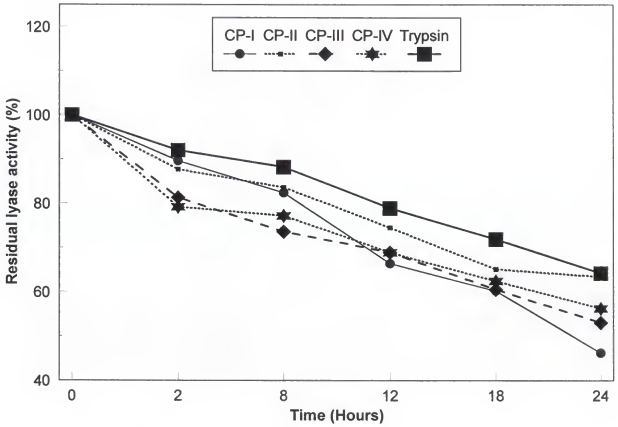

CP-I
CP-II
CP-III
CP-IV
Control
Residual activity (%)
Time (Hours)
0
20
40
60
80
100
0
15
30
45
60
75

Figure 28. Effect of crawfish proteases and trypsin on pectate lyase c from *Erwinia chrysanthemi* EC16. The ratio (w/w) of proteases to pectate lyase c was 1:40.


CP-I CP-II CP-III CP-IV Trypsin
Residual lyase activity (%)
120
100
80
60
40
0
2
8
12
18
24
Time (Hours)

Selective Cleavage of Native PLc by Proteases

Native Pectate lyase c was digested by trypsin and the four crawfish proteases to investigate how the proteases attack proteins. Pectate lyase c was chosen as a model protein to evaluate selective proteolysis because it has a well characterized stricture and amino acid sequence (Hurlbert, 1996; Hurlbert et al., 1996). Samples were collected with time and analyzed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometer. Mass spectrograms are presented in Figures 29-33 and the masses of the peptide fragments found are summarized in Tables 11-15.

Using the computer program PAWS, it is possible to estimate the amino acid sequences of the conserved peptides present in the reaction mixtures based upon their masses. Hurlbert (1996) has studied trypsin cleavage of native pectate lyase c. Lysine at 172 or 189 seems to be susceptible to trypsin cleavage while lysine 43 seems to be cleaved by the crawfish proteases. These positions are marked by the arrows in the Rasmol image of PLc in Figure 34. Peptides generated during proteolysis of pectate lyase by the four crawfish proteases shows similar patterns and indicates a common mechanism for each isozyme. The cleavage patterns were different from those obtained with the action of bovine trypsin on pectate lyase.

Figure 29. MALDI-TOF mass spectrograms of the 0 (A), 2 (B) and 12 (C) hour digest of native PLc using trypsin. Sinapinic acid was used as the matrix, mixed 1:1 with sample and placed on a sample pin for analysis on a Vestec Research H Matrix Assisted Laser Desorption Ionization Time of Flight mass spectrometer. Data were analyzed with GRAMS/386 software package (Galactic Industries) running on a Zeos 486/33 computer.

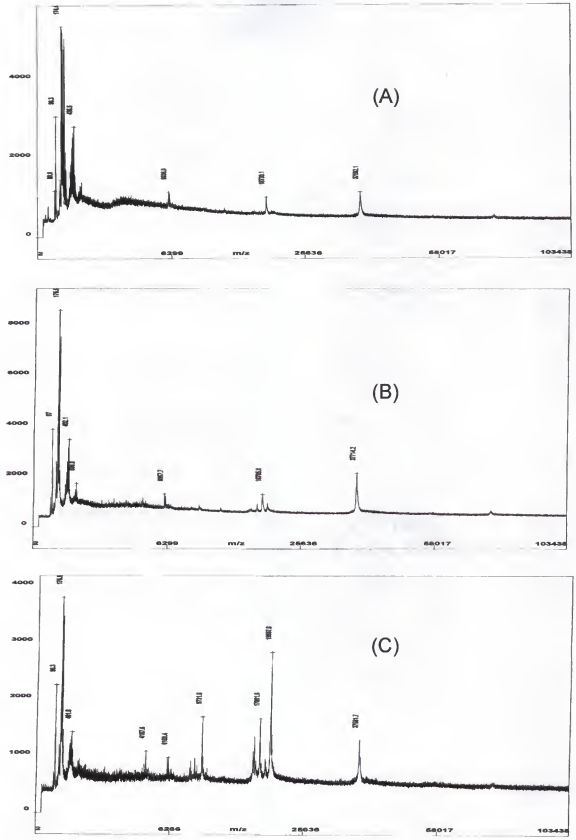

(A)
(B)
(C)
m/z
6299
25636
58017
103436

Table 11. Peptides generated during trypsin proteolysis of native PLc

	PLc Mass Fragments (Daltons)		
0 hour:	18730[a]	37592[b]	
2 hour:	18785[a]	37714[b]	
12 hours:	4188	9732	17882
	19598	37582[a]	

[a] Peptide masses representing the M/2Z signals of the PLc peptide fragments.
[b] Peptide masses representing the M/Z signals of the PLc peptide fragments.

Figure 30. MALDI-TOF mass spectrograms of the 0 (A), 2 (B) and 12 (C) hour digest of native PLc using CP-I. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

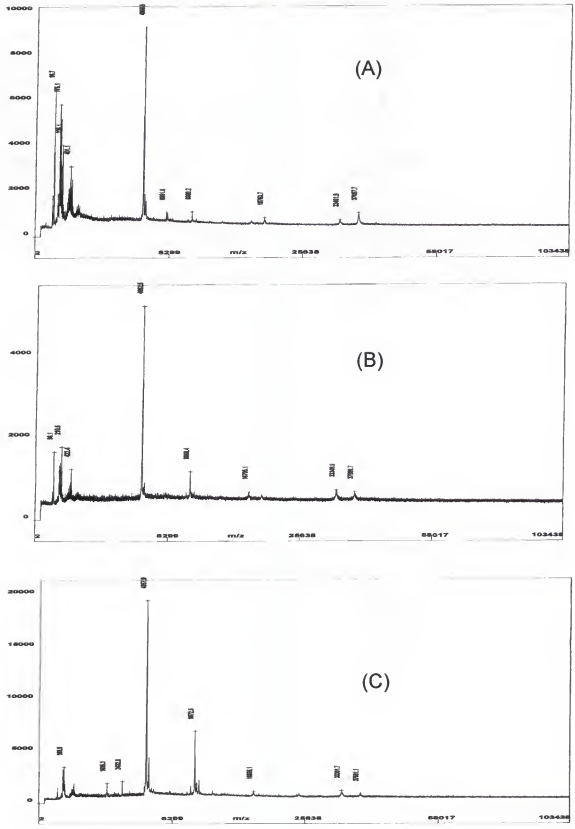

(A)
(B)
(C)
m/z

Table 12. Peptides generated during CP-I proteolysis of native PLc

	PLc Mass Fragments (Daltons)		
0 hour:	4067 33452	8690 37658[b]	18764[a]
2 hours:	4053 33350	8668 37600[b]	16705
12 hours:	1605 8673 37581[b]	2433 16655	4058 33302

[a] Peptide masses representing the M/2Z signals of the PLc peptide fragments.
[b] Peptide masses representing the M/Z signals of the PLc peptide fragments.

Figure 31. MALDI-TOF mass spectrograms of the 0 (A), 2 (B) and 12 (C) hour digest of native PLc using CP-II. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

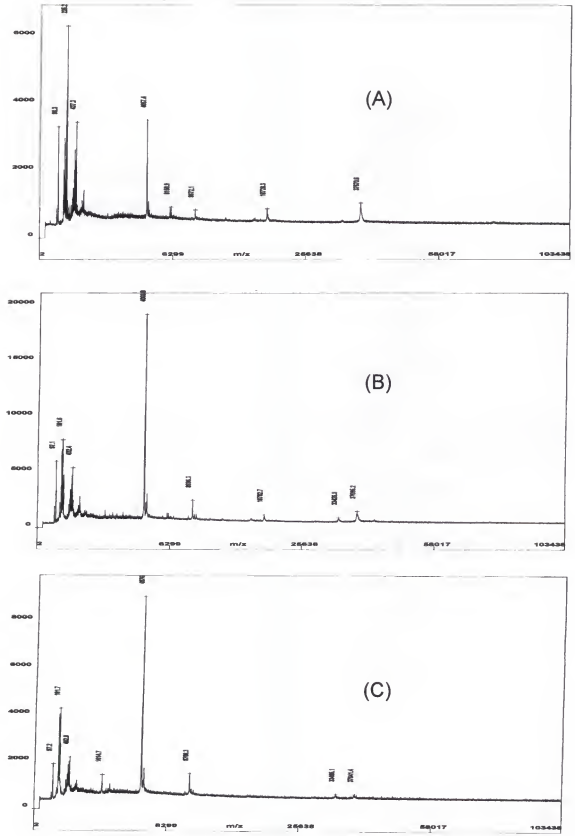

(A)
(B)
(C)
m/z

Table 13. Peptides generated during CP-II proteolysis of native PLc

	PLc Mass Fragments (Daltons)		
0 hour:	4057 37571[b]	8672	18728[a]
2 hours:	4069 33427	8696 37695[b]	18783[a]
12 hours:	1615 33485	4076 37841[b]	8708

[a] Peptide masses representing the M/2Z signals of the PLc peptide fragments.
[b] Peptide masses representing the M/Z signals of the PLc peptide fragments.

Figure 32. MALDI-TOF mass spectrograms of the 0 (A), 2 (B) and 12 (C) hour digest of native PLc using CP-III. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

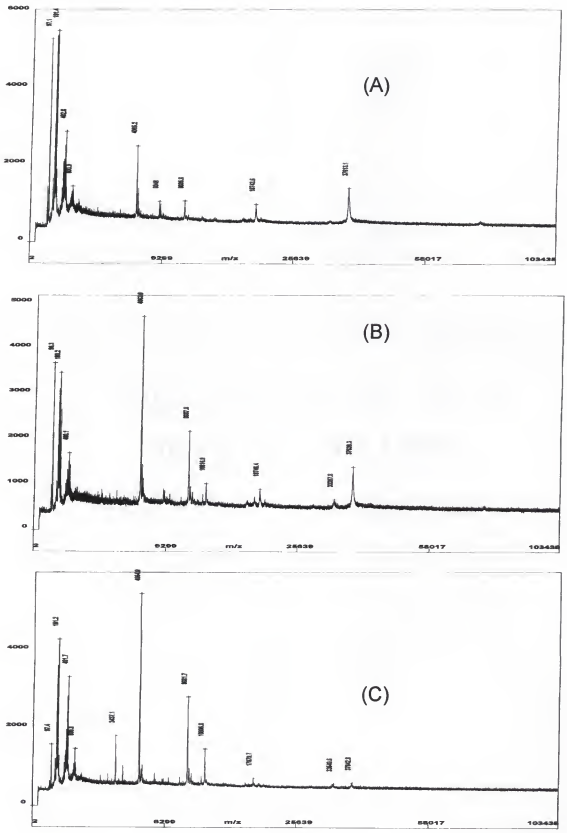
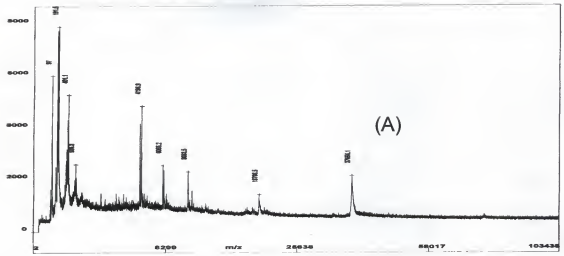

(A)
(B)
(C)
m/z

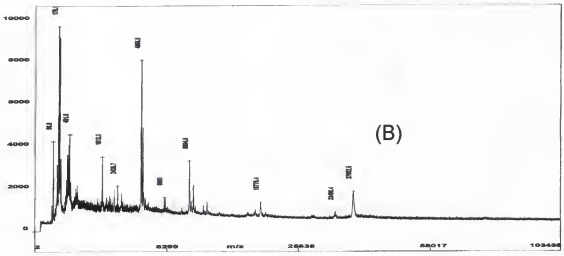
Table 14. Peptides generated during CP-III proteolysis of native PLc.

	PLc Mass Fragments (Daltons)		
0 hour:	4065 37613[b]	8686	18744[a]
2 hours:	4064 18747[a]	8688 33388	10817 37639[b]
12 hours:	2437 10806 37843[b]	4065 17880	8682 33541

[a] Peptide masses representing the M/2Z signals of the PLc peptide fragments.
[b] Peptide masses representing the M/Z signals of the PLc peptide fragments.

Figure 33. MALDI-TOF mass spectrograms of the 0 (A), 2 (B) and 12 (C) hour digest of native PLc using CP-IV. Samples were prepared and data collected and analyzed by the methods described in Figure 29.


(A)
m/z


(B)
m/z

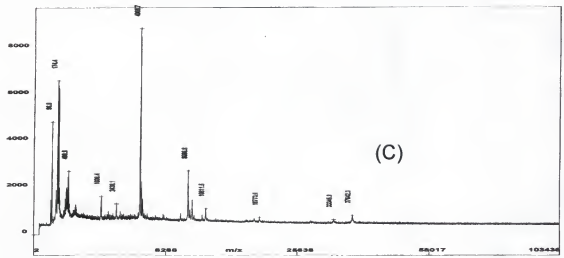
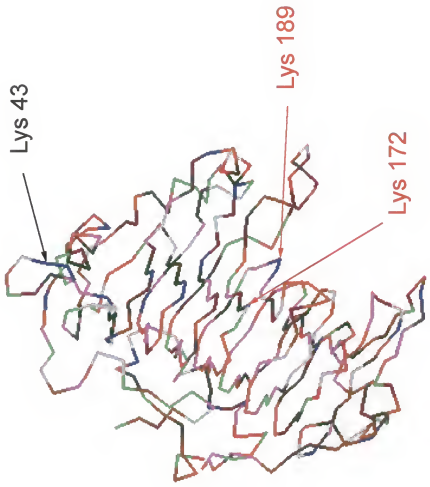

(C)
m/z

Table 15. Peptides generated during CP-IV proteolysis of native PLc.

	PLc Mass Fragments (Daltons)		
0 hour:	4197 37656[b]	8694	18767[a]
2 hours:	1613 8695 37653[b]	2436 18775[a]	4067 33498
12 hours:	1609 8687 33346	2438 10812 37642[b]	4066 18774[a]

[a] Peptide masses representing the M/2Z signals of the PLc peptide fragments.
[b] Peptide masses representing the M/Z signals of the PLc peptide fragments.

Figure 34. Rasmol image of PLc showing probable cleavage sites for trypsin at either Lysine 172 or Lysine 189 while the probable cleavage site for the crawfish proteases is Lysine 43.


Lys 43
Lys 189
Lys 172

Cleavage of Urea Denatured PLc by proteases

Pectate lyase c was denatured with 2, 4 and 8 M urea for 6 hours and digested overnight by trypsin and the four crawfish proteases. Mass spectrograms for 4 M urea treated PLc are presented in Figures 35-39. Peptides generated during proteolysis of urea treated PLc are summarized in Table 16.

Trypsin seemed to be more resistant to urea than the crawfish proteases because trypsin proteolysis of 8 M urea treated PLc generated more peptides than the crawfish proteases. CP-III and CP-IV were more resistant to urea than CP-I and CP-II. In the 2 and 4 M urea treated PLc digestions, crawfish proteases were not affected by urea when compared to the digestions by trypsin. The peptides of mass 4386-4390 generated by trypsin proteolysis were not generated by the crawfish proteases. The peptides of mass 4652-4693 were generated by proteolysis of the crawfish proteases but not by trypsin proteolysis. This indicates that the cleaving pattern of crawfish proteases on denatured PLc is different from that of trypsin.

Figure 35. MALDI-TOF mass spectrogram of 12 hour digest of 4 M urea treated PLc using trypsin. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

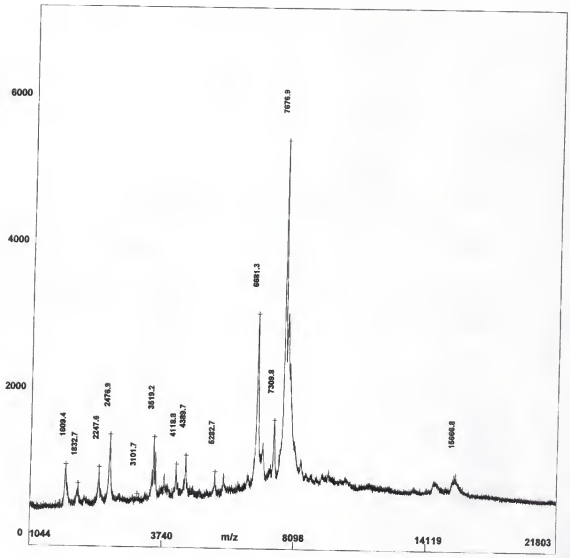

6000
4000
2000
0
1044
3740
m/z
8098
14119
21803
1609.4
1832.7
2247.6
2476.8
3101.7
3519.2
4118.6
4365.7
6282.7
6681.3
7309.8
7676.9
15668.8

Figure 36. MALDI-TOF mass spectrogram of 12 hour digest of 4 M urea treated PLc using CP-I. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

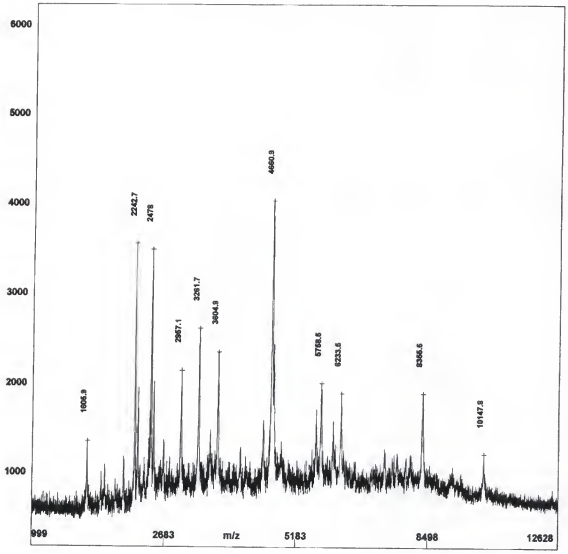

6000
5000
4000
3000
2000
1000
1656.9
2242.7
2478
2967.1
3251.7
3604.3
4660.9
5785.6
6233.6
8366.6
10147.8
999
2683
m/z
5183
8498
12628

Figure 37. MALDI-TOF mass spectrogram of 12 hour digest of 4 M urea treated PLc using CP-II. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

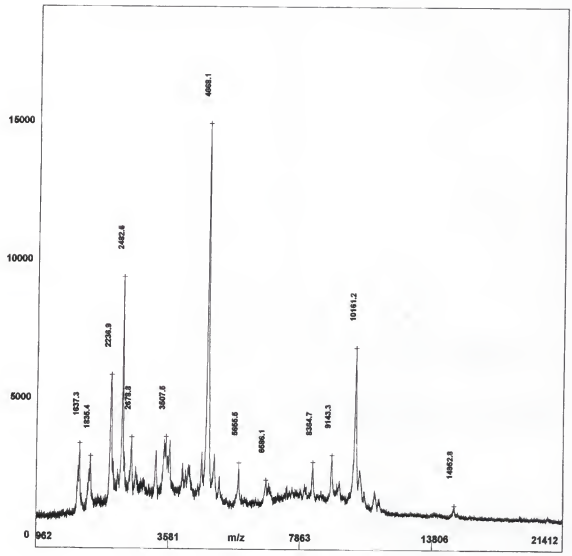

15000
10000
5000
0
962
3581
m/z
7863
13806
21412
1637.3
1826.4
2238.9
2482.6
2678.3
3607.8
4668.1
6666.6
6686.1
8354.7
9143.3
10161.2
14852.8

Figure 38. MALDI-TOF mass spectrogram of 12 hour digest of 4 M urea treated PLc using CP-III. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

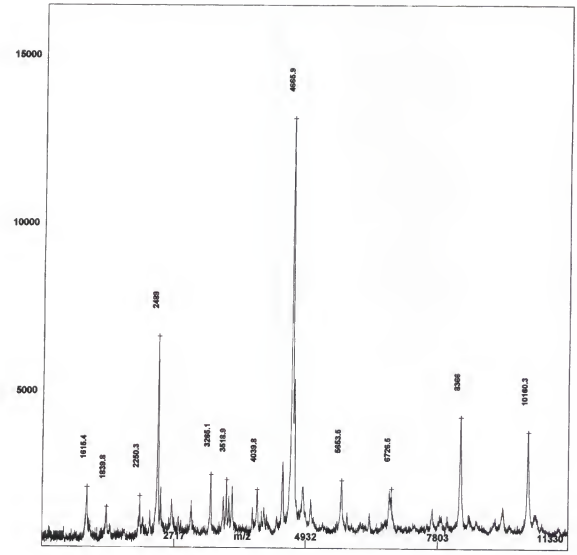

15000
10000
5000
1616.4
1838.8
2280.3
2469
3266.1
3516.0
4039.8
4666.9
5563.6
6726.5
8366
10160.3
m/z
2717
4932
7803
11350

Figure 39. MALDI-TOF mass spectrogram of 12 hour digest of 4 M urea treated PLc using CP-IV. Samples were prepared and data collected and analyzed by the methods described in Figure 29.

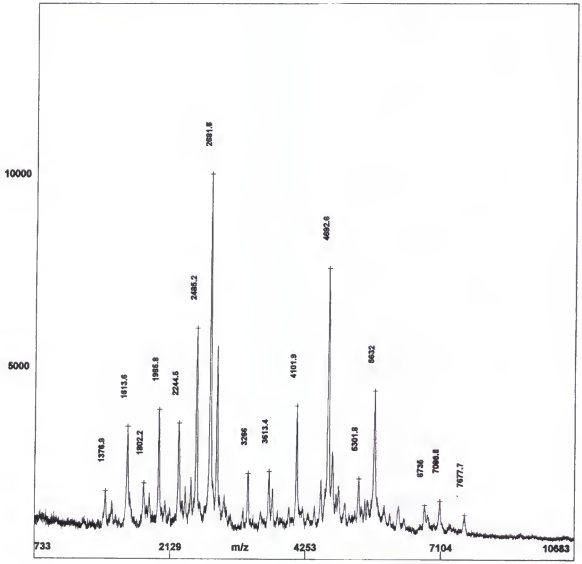

10000
5000
1378.9
1613.6
1802.2
1985.9
2244.5
2485.2
2691.8
3298
3513.4
4101.9
4682.9
5301.8
6532
6736
7086.8
7677.7
733
2129
m/z
4253
7104
10683

Table 16. Peptides generated during the proteolysis of urea denatured PLc[a] by trypsin and crawfish proteases

MALDI-TOF Observed Mass Range	Trypsin			CP-I			CP-II			CP-III			CP-IV		
	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]
1244	X														
1373- 1377														X	X
1604- 1637	X	X	X		X	X		X	X	X	X		X	X	X
1802- 1840	X	X				X		X			X			X	
1958- 1986	X													X	X
2236- 2250	X	X			X		X	X			X		X	X	X
2330			X												
2409- 2439	X						X								
2475- 2489		X	X		X	X		X	X	X	X	X	X	X	X
2660- 2688			X			X		X	X			X		X	X
2779															X

[a] PLc was denatured by urea for 6 hours at 37°C and then was digested by proteases for 12 hours at room temperature.
[b] The ratio of PLc to proteases was 64:1(w/w).
[c] The ratio of PLc to proteases was 32:1(w/w).
[d] The ratio of PLc to proteases was 16:1(w/w).

Table 16. (Continued)

MALDI-TOF Observed Mass Range	Trypsin			CP-I			CP-II			CP-III			CP-IV		
	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]
2942- 2968					X	X						X	X		
3066- 3102	X	X													
3253- 3268					X	X			X		X	X		X	
3468															X
3507- 3519	X	X						X			X				
3600- 3617					X	X			X			X	X	X	
3905	X														
4039- 4076											X				
4101- 4187		X												X	
4386- 4390		X	X												
4563	X														

[a] PLc was denatured by urea for 6 hours at 37°C and then was digested by proteases for 12 hours at room temperature.
[b] The ratio of PLc to proteases was 64:1(w/w).
[c] The ratio of PLc to proteases was 32:1(w/w).
[d] The ratio of PLc to proteases was 16:1(w/w).

Table 16. (Continued)

MALDI-TOF Observed Mass Range	Trypsin			CP-I			CP-II			CP-III			CP-IV		
	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]
4652 -4693					X	X		x	x	X	X	X	X	X	
5042													x		
5282- 5302		X												X	
5506- 5563			X			X							X		
5622- 5658	X							X	X		X			X	X
5742-5774					X	X						X			
5953															X
6228- 6250					X	X						X			
6308															X
6586								X							
6637- 6682		X	X							X					X

[a] PLc was denatured by urea for 6 hours at 37°C and then was digested by proteases for 12 hours at room temperature.
[b] The ratio of PLc to proteases was 64:1(w/w).
[c] The ratio of PLc to proteases was 32:1(w/w).
[d] The ratio of PLc to proteases was 16:1(w/w).

Table 16. (Continued)

MALDI-TOF Observed Mass Range	Trypsin			CP-I			CP-II			CP-III			CP-IV		
	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]
6726- 6735											X			X	
7089														X	
7304- 7342		X	X						X						
7645- 7680		X	X			X			X			X		X	X
7775- 7813	X									X					
8003															X
8345- 8369	X				X	X		X	X		X	X			X
8690															X
9142- 9144								X	X						
9213	X														
10053	x														

[a] PLc was denatured by urea for 6 hours at 37°C and then was digested by proteases for 12 hours at room temperature.
[b] The ratio of PLc to proteases was 64:1(w/w).
[c] The ratio of PLc to proteases was 32:1(w/w).
[d] The ratio of PLc to proteases was 16:1(w/w).

Table 16. (Continued)

MALDI-TOF Observed Mass Range	Trypsin			CP-I			CP-II			CP-III			CP-IV		
	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]	8M[b]	4M[c]	2M[d]
10147-10162					X			X	X	X	X				
11023-11026	X									X					
11743	X														
14953								X							
15669		X													

[a] PLc was denatured by urea for 6 hours at 37°C and then was digested by proteases for 12 hours at room temperature.
[b] The ratio of PLc to proteases was 64:1(w/w).
[c] The ratio of PLc to proteases was 32:1(w/w).
[d] The ratio of PLc to proteases was 16:1(w/w).

CONCLUSIONS

Protease from the pyloric ceca of stomachless fish, mullet, was partially purified. The enzyme was able to inactivate native orange pectinesterase.

Four proteases from the hepatopancreas of the stomachless crustacean crawfish were purified and characterized. The enzymes were inhibited by protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), aprotinin, and tosyl lysine chloromethyl ketone (TPCK) but not by the chymotrypsin inhibitor tosyl phenylalanine chloromethyl ketone (TPCK). Therefore, crawfish proteases could be considered as serine-type proteases, and classified as trypsin-like, lacking chymotrypsin activity.

Two crawfish proteases were activated by mercury which is known as an inhibitor for proteases while the other two proteases were inhibited. This indicates that these two proteases might be unique novel type enzymes. Future work is needed to understand this unusual phenomenon.

Immunological analysis showed strong cross reactivity between the four enzymes, indicating they shared structural components which constituted common epitopes for polycloned antibodies.

Also, digestion using pectate lyase c as a target protein showed that the crawfish enzymes cleave proteins in similar patterns, indicating the four crawfish proteases could be considered as isozymes. However, their cleaving patterns were different from bovine trypsin.

Proteases from crawfish could inactivate native enzymes such as orange pectinesterase, tomato pectinesterase and pectate lyase. The proteases could be used in inactivating the deleterious enzymes in food processing without any heat treatment. These enzymes might be a better alternative to trypsins in industrial applications.

The findings of this study on proteases from stomachless aquatic organisms are very useful in understanding protease mechanisms and function as well as their potential for industrial applications.

Since the yield of the enzyme by conventional methods was low, cloning the enzyme for mass production is encouraged for future work.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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